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The studies performed under this grant indicate that abnormalities in the expression of the cell cycle control proteins cyclin D1, cyclin E and $p27^{Kip1}$ frequently occur during the development of breast cancer. They also revealed for the first time the existence of homeostatic feedback control mechanisms between these critical regulators of the cell cycle. These mechanisms may explain the paradoxical increase in expression of the $p27^{Kip1}$ protein in some breast cancer cells. These studies also revealed that increased expression of cyclin D1 or cyclin E can have complex phenotypic effects on cell proliferation, apoptosis and responses to TGFβ. Our studies on $p27^{Kip1}$ suggest that therapeutic strategies that further increase the level of expression of this protein, or mimic its activity, might provide a novel approach to breast cancer chemoprevention and therapy.

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I. Bernard Weinstein 8/6/98

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THE ROLE OF CYCLIN D1 OVEREXPRESSION IN BREAST CANCER PROGRESSION

(5) INTRODUCTION

Nature of the Problem. Although there is considerable evidence that the majority of human breast cancers are caused by environmental factors (including dietary factors) and reproductive factors, rather than hereditary factors, the specific causes of human breast cancer are not known with certainty. Furthermore, although exciting progress has been made in identifying mutations and or aberrant expression of cellular oncogenes and tumor suppressor genes in human breast cancers, the precise mechanisms responsible for the uncontrolled proliferation of breast cancer cells, the apparent genomic instability of malignant breast tumors, and the often relentless course of tumor progression, are poorly understood at the present time.

Background. Cyclins are a recently identified family of proteins that regulate the passage of cells through the G1, S, G2 and M phases of the cell cycle (for review see 1-5). These proteins complex with specific cyclin-dependent serine-and threonine-protein kinases (CDKs), thereby regulating the activity of these kinases. This process is further modulated by the phosphorylation and dephosphorylation of CDK proteins by specific pr54otein kinases and phosphatases, and by specific inhibitory proteins called CDKIs. At least 7 distinct cyclin genes have been identified in the human genome and at least six CDKs (CDK 1-6) form complexes with these cyclins. Based on their conserved sequence motifs with cyclins in other species and their patterns of expression and apparent functional roles during the cell cycle, they are grouped into three categories: G1 cyclins, including cyclins D1-3, and E; the S-phase cyclin, cyclin A; and G2/M phase cyclins, cyclins A, B1 and B2. Cyclin B is the best characterized mammalian cyclin. It complexes with CDK1 (CDC2) to regulate both mitotic entry and exit. It is not known whether cyclin B1 and B2 are interchangeable. After stimulating quiescent cells by growth factors, cyclin D1 is expressed maximally during mid to late G1, although it appears to be expressed at a constant level in continuously dividing cells. Cyclin D1, and cyclins D2 and D3, associate with CDKs 4 and 6 thereby activating their activities. This leads to phosphorylation and inactivation of the tumor suppressor protein Rb, thus causing activation of the transcription factor E2F which enhances S phase progression. It is not known whether all of the biologic effects of cyclin D1 are mediated via the Rb protein. Cyclin E acts in late G1 by activating CDK2 and cyclin A acts in the S and G2/M phases by activating CDK2.

Several lines of evidence implicate the role of cyclins D1 and E in human breast cancer. 1) The cyclin D1 gene originally termed Prad 1, is located at chromosome 11q13. We and other investigators have demonstrated amplification and/or increased expression of cyclin D1 in a subset of primary human breast cancer cell lines (6-9). 2) Increased expression and deregulation in the expression of cyclin E have been described in human breast tumors and cell lines (1, 2, 4, 10, 11). It is of interest that the increased expression of cyclin D1 and cyclin E do not always correlate with amplification of the corresponding genes.

Our laboratory has previously described amplification and/or increased expression of cyclin D1 in human tumors of the esophagus, liver, and colon (3, 4, 12, 13). We have also demonstrated that stable overexpression of cyclin D1 shortens the G1 phase of the cell cycle and

enhances malignant cell transformation (5). These studies from our laboratory, coupled with the other evidence (cited above) of abnormalities in cyclins in human breast cancers, provide the basis of this grant.

Purpose of the present work. The overall purpose of this work is to examine the hypothesis that abnormalities in the expression of cyclin D1 and related cell cycle control genes play important roles in multistage breast carcinogenesis by enhancing the process of tumor progression. These studies might provide new biomarkers and diagnostic tools to more precisely detect and stage breast tumors. This approach could also lead to the development of agents that inhibit the action of specific cyclins or cyclin-dependent protein kinases in human tumors, and thus lead to novel strategies for breast cancer chemoprevention and therapy. If abnormalities in the expression of cyclin genes enhance genomic instability, as suggested by our recent studies (14), then such inhibitors might specifically block the progress of tumor progression and the emergence of hormone independent and drug resistant variants of breast tumors.

Methods of approach. As discussed above, the cyclin D1 gene is frequently amplified and/or overexpressed in primary human breast cancers and breast cancer cell lines, but the functional and prognostic significance of this finding is not known. We are using well defined normal human mammary epithelial and human breast cancer cell culture systems to analyze the role of cyclin D1 in cell cycle control, gene expression and amplification, cell transformation and tumorigenicity. Similar studies are also being done with cyclin E. A major strategy employed in our studies is to utilize gene transfer methods to develop derivatives of normal mammary epithelial cells that stably overexpress either cyclin D1, cyclin E or $p27^{Kip1}$ and then examine possible effects on growth control, differentiation and various cell cycle parameters (6, 9, 11, 15, 18).

The studies supported by this grant have employed several experimental systems, including: 1) carcinogen-induced rat mammary tumors; 2) cell lines derived from either normal mammary epithelium or breast carcinomas; 3) genetically engineered derivatives of these cell lines that stably overexpress cyclin D1, cyclin E or $p27^{Kip1}$; and, 4) a series of primary human breast carcinomas studied by immunohistochemistry.

(6) BODY (for the complete experimental details see reprints in the Appendix)

(a) Studies on this grant carried out in 1994-1996

In our previous annual progress reports we provided the first evidence that there is a marked increase in the expression of cyclin D1 in NMU-induced primary rat mammary tumors (16). Significant but less striking increases in the expression of cyclin E, cyclin A, CDK2 and CDK4 were also seen (16). We also described amplification and increased expression of cyclin D1 in several human breast cancer cell lines (6). We found that stable overexpression of an exogenous cyclin D1 cDNA in the HBL-100 and HC11 mammary epithelial cell lines, inhibited growth and enhanced apoptosis (6, 9). An unexpected finding was that increased expression of

cyclin D1 in HBL-100, HC11 and MCF-10F cells was associated with increased expression of the cell cycle inhibitory protein $p27^{Kip1}$ (9). These findings provide evidence for a homeostatic feed-back inhibitor loop between cyclin D1 and $p27^{Kip1}$ (9). In a separate study we found that overexpression of cyclin E, which frequently occurs in human breast cancers, in HC11 cells also inhibited growth and induced expression of $p27^{Kip1}$ (11). The latter findings were subsequently examined in greater detail, as described below.

(b) Studies carried out in 1996 - 1997

(1) Effects of cyclin E overexpression on cell growth and response to transforming growth factor β depend on cell context and $p27^{Kip1}$ expression (15).

Human breast tumors often display increased expression and dysregulation of cyclin E (10, 11), suggesting that this might contribute to their abnormal growth. Cyclin E binds to and activates CDK2 and this complex plays a critical role in cell cycle progression, acting in late G1 after cyclin D1. However, in 1996 we reported that overexpression of a human cyclin E cDNA in the nontransformed mouse mammary epithelial cell line HC11 resulted in increased expression of the cell cycle-inhibitory protein $p27^{Kip1}$ and inhibition of cell growth (11). To further address the significance of this finding and the role of cell context, in the present study we analyzed, in parallel, the effects of cyclin E overexpression in two fibroblast cell lines (Rat1 and NIH3T3) and three nontumorigenic mammary epithelial cell lines (the human mammary cell lines 184B5 and MCF-10F and the mouse mammary cell line HC11).

Derivatives of the above described 5 cell lines were obtained by infecting the cells with retrovirus particles containing a cyclin E cDNA sequence and selection for hygromycin resistant cells, as previously described (11). Vector control cells infected with retrovirus particles lacking the cyclin E sequence (11) were also prepared and always analyzed in parallel with the cyclin E overexpressor cells for several phenotypic properties. The details for the procedures used for cell culture, the construction of the retrovirus vectors, viral transduction, growth curves, assays for anchorage-independent growth, flow cytometry for cell cycle kinetics, western blot analyses, and assays for cyclin/CDK kinase activities have been described in our previous publications (6, 9, 11).

To further address the role of cyclin E in mammary tumorigenesis, and the effects of cell context, we analyzed in parallel the effects of cyclin E overexpression in two fibroblast cell lines (Rat1 and NIH3T3) and three nontumorigenic mammary epithelial cell lines (the human mammary cell line 184B5 and MCF-10F and the mouse mammary cell line HC11). We found that increased expression of cyclin E was associated with increased cyclin E-associated kinase activity in Rat1, NIH3T3, and MCF-10F cells but not in HC11 and 184B5 cells. It is of interest that the derivatives of the latter two cell lines showed increased expression of $p27^{Kip1}$ and inhibition of cell growth. There was a shortening of the G_1 phase in the derivatives of the Rat1 and MCF-10F cells but not in the derivatives of the other three cell lines. Contrary to a previous hypothesis, overexpression of cyclin E was not able to confer anchorage-independent growth in any of these cell lines. However, overexpression of cyclin E was associated with increased

resistance to transforming growth factor β-mediated growth inhibition in the 184B5 and HC11 cells and a decrease in transforming growth factor β stimulation of the Rat1 and NIH3T3 fibroblasts.

Thus, overexpression of the same cyclin E cDNA has cell type-specific effects on various growth parameters. We previously provided evidence that in some mammary epithelial and breast cancer cell lines there is a homeostatic feed-back loop between cyclin D1 and $p27^{Kip1}$ (9). The present study provides evidence that there is also a homeostatic feed-back loop between cyclin E and $p27^{Kip1}$, which is also cell context dependent. Our results are not confined to cell culture systems since, although $p27^{Kip1}$ is a putative tumor suppressor, in studies described below we found that $p27^{Kip1}$ is also expressed at relatively high levels in about 50% of primary human breast cancers.

(2) Overexpression of Cyclin D1 in Primary Human Breast Cancers and Correlations with Various Clinical and Pathologic Parameters

In view of our previous findings on cyclin D1 overexpression in NMU-induced rat mammary tumors and in human mammary epithelial and breast cancer cell lines, it was of interest to examine cyclin D1 expression in a series of various types of primary human breast cancers and search for possible correlations with various clinical and pathologic parameters. Therefore, we determined the levels of cyclin D1 expression in 140 cases of primary breast cancer by immunostaining of formalin fixed-parrafin embedded tissue, using an immunoperoxidase technique. The immunostaining method, controls for specificity and the scoring procedure were essentially the same as previously employed by our laboratory in a study on cyclin D1 in human colon cancer (13). Staining intensities of 2 or 3 were considered positive for overexpression since 0 or 1 intensities, but never 2 or 3, were seen in normal adjacent tissue. In addition, at least 5% of the tumor cells in a given case had to show overexpression for the case to be considered positive.

The overall positive rate for cyclin D1 overexpression in this group of cases was 68%. We found a significant correlation between cyclin D1 overexpression and the following parameters: estrogen receptor positivity, high tumor grade, aneuploidy, high proliferation index and low expression of HER 2/c-neu. Follow-up information on these cases in terms of recurrence of disease, response to therapy and survival is not yet available. Therefore, the clinical significance of these findings with respect to prognosis remains to be determined. Nevertheless, it is apparent from our studies, and those by other investigators, that overexpression of cyclin D1 is one of the most frequent characteristics of human breast cancer. An abstract describing these results was published in the Proceedings of the American Society of Clinical Oncology 1998, and a manuscript describing these results is in preparation.

(c) Studies carried out during the past year, 1997 - 1998

(1) Deregulated Expression of $p27^{Kip1}$ in Human Breast Cancers

The $p27^{Kip1}$ protein belongs to a family of cyclin dependent kinase-inhibitory proteins that are negative regulators of cell cycle progression and have been proposed as candidate tumor suppressor genes. However, the $p27^{Kip1}$ gene is only rarely mutated in human primary breast carcinomas and breast cancer cell lines. To further address the role of $p27^{Kip1}$ in the development of human tumors, we determined by Western blot analysis the levels of expression of the $p27^{Kip1}$ protein in a series of human cancer cell lines and found that this protein is expressed at high levels in many of these cell lines, even during exponential growth (17). The levels of $p27^{Kip1}$ were significantly associated with the levels of cyclins D1 and E. In contrast to the high level of $p27^{Kip1}$ in breast cancer cell lines, three cell lines established from normal mammary epithelium expressed low levels of this protein. Cell synchronization studies demonstrated deregulation of the expression of $p27^{Kip1}$ throughout the cell cycle in two breast cancer cell lines but normal regulation in a normal mammary epithelial cell line. Immunohistochemical studies on $p27^{Kip1}$ expression in 52 primary human breast cancers indicated that this protein was also expressed at relatively high levels in 44% of the tumor samples, but it was barely detectable or undetectable in the remaining 56% of the samples (17). Additional studies are required to determine why some breast cancer cells express relatively high levels of $p27^{Kip1}$ despite its known role as an inhibitor of cell cycle progression.

(2) <u>Overexpression of p27Kip1 Inhibits the Growth of Both Normal and Transformed Human Mammary Epithelial Cells</u>

Overexpression of $p27^{Kip1}$ has been reported to induce cell cycle arrest in a variety of cell lines, although most of the previous studies employed fibroblasts rather than epithelial cells. Nevertheless, as described above we found increased expression of this inhibitory protein in human breast cancer cell lines and a subset of primary human breast carcinomas (17). The increased expression of $p27^{Kip1}$ in these cancer cells is especially intriguing because several studies have also indicated that the $p27^{Kip1}$ gene is usually not mutated in breast cancers and, therefore, they overexpress a wild type protein.

We hypothesized that the increased levels of $p27^{Kip1}$ in breast cancer cells might reflect the existence of a homeostatic regulatory mechanism that protects the cancer cells from potentially toxic effects of increased expression of cyclin E and/or cyclin D1. Indeed, as described above we observed a significant association between increased expression of $p27^{Kip1}$, cyclin E or cyclin D1 in human breast cancer cell lines (17). The paradoxical increase in $p27^{Kip1}$ in breast cancer cells might indicate that mammary epithelial cells are inherently resistant to the inhibitory effects of $p27^{Kip1}$ or that cyclin/CDK complexes in breast cancer cells are refractory to the inhibitory activity of $p27^{Kip1}$ due to mutations in one (or more) components of these complexes. Therefore, it was of interest to examine the phenotypic effects of expressing an exogenous $p27^{Kip1}$ in both normal and tumor-derived human mammary epithelial cells.

In a study carried out during the past year (18) we demonstrated that, as previously reported for other cell types, overexpression of $p27^{Kip1}$ does inhibit the growth of normal human mammary epithelial cells. In fact, overexpression of an exogenous $p27^{Kip1}$ cDNA in the normal non-tumorigenic human mammary epithelial cell line MCF10F was associated with lengthening of the G1 phase of the cell cycle, a longer doubling time, a decreased saturation density and a decreased plating efficiency (Table 1). As expected, these phenotypic effects were associated

with a marked inhibition of cyclin E-associated kinase activity.

Despite its rapid in vitro growth and in vivo tumorigenicity, MCF-7 cells express a high level of $p27^{Kip1}$ (17,18). Nevertheless, a further increase in the cellular level of $p27^{Kip1}$ due to ectopic expression of $p27^{Kip1}$ was also able to inhibit the growth of this cell line (18). Thus, the $p27^{Kip1}$-overexpressing derivatives of the MCF7 cells displayed an increase in the percent of cells in the G1 phase of the cell cycle, a longer doubling time, a decreased saturation density and a decreased plating efficiency (Table 1). Furthermore, the anchorage-independent growth and the in vivo tumorigenicity of MCF7 cells were significantly reduced, although not completely abolished (Table 1), by the increased expression of $p27^{Kip1}$. Cyclin E- associated kinase activity was also markedly decreased in these derivatives, when compared with vector control MCF7 cells.

Overexpression of $p27^{Kip1}$ had different effects on the levels of expression of the cyclin D1 protein in the normal MCF10F and MCF7 breast cancer cell lines (18). The levels of the cyclin D1 protein were reduced in the $p27^{Kip1}$ -overexpressing derivatives of the MCF10F cells, when compared with the vector control MCF10F cells. The mechanism responsible for this decrease is not known. It could simply reflect the growth inhibition seen in these derivatives. However, the level of the cyclin E protein was slightly increased in the $p27^{Kip1}$ overexpressing MCF10F cells. The latter effect might reflect an attempt of the cells to override the growth inhibition caused by the increased level of $p27^{Kip1}$. It might also simply be a consequence of reduced turnover of the cyclin E protein, since cyclin E is degraded following its phosphorylation by the cyclin E/Cdk2 complex, and this kinase activity is inhibited in these cells.

Curiously, overexpression of $p27^{Kip1}$ in the breast cancer - derived MCF7 cells was associated with increased levels of both the cyclin D1 and cyclin E proteins (18). The increased level of cyclin E might, again, be a consequence of reduced turnover due to reduced phosphorylation by the cyclin E/Cdk2 complex. More intriguing is the increased expression of cyclin D1 since this did not occur in the derivatives of MCF10F cells. The increased expression of cyclin D1 and cyclin E in the $p27^{Kip1}$ -overexpressing derivatives of MCF7 cells may also reflect an adaptive mechanism in which the tumor cell attempt to override the growth inhibitory activity of the increased level of $p27^{Kip1}$. Indeed, increased expression of cyclin E has been shown to rescue cells from $p27^{Kip1}$ -mediated growth inhibition. Western blot analyses failed to detect any change in the levels of expression of cyclin A, Cdk2, Cdk4 or $p21^{Cip1}$ in the $p27^{Kip1}$-overexpressing derivatives of MCF10F or MCF7 cells (data not shown).

We previously observed a reciprocal phenomenon, in which ectopic overexpression of cyclin D1 or cyclin E in mammary epithelial cells is associated with increased expression of $p27^{Kip1}$ (9,11,15). Increased levels of endogenous cyclin D1 and cyclin E also correlate with increased levels of $p27^{Kip1}$ in breast cancer cell lines (17). Furthermore, there is a significant association between the levels of $p27^{Kip1}$ and cyclin D1 in primary breast carcinomas (17). It is not clear whether the increase in cyclins D1 and E or the increase in $p27^{Kip1}$ occur first during tumor development.

Taken together, these findings provide evidence for the existence in mammalian epithelial cells of a complex homeostatic network of feedback loops that maintains an optimum balance between positive and negative regulators of the G1 to S transition of the cell cycle. It appears that at least some components of this network can be retained in breast cancer cells, presumably because this provides a growth and/or survival advantage. However, the present study

demonstrates that breast cancer cells are unable to counteract a marked or abrupt increase in the level of expression of $p27^{Kip1}$. In fact, despite the increased expression of cyclin D1 and cyclin E, the $p27^{Kip1}$ overexpressing derivatives of MCF7 cells were inhibited in both their growth and tumorigenicity (Table 1). These results suggest that strategies which would increase cellular levels of $p27^{Kip1}$, or mimic its effects, might be useful in cancer therapy, since they would inhibit the growth and tumorigenicity of cancer cells, even in the presence of high endogenous levels of cyclin D1 and/or cyclin E. Several growth inhibitory factors, such as TGF-β, contact inhibition IFN-γ, IFN-β, cAMP and rapamycin may function, at least in part, by inducing the expression or enhancing the activity of $p27^{Kip1}$ (for review see 18). These observations suggest novel strategies for the therapy of breast cancer.

Table 1 Effects of $p27^{Kip1}$ overexpression on cell cycle distribution and growth properties in normal human mammary epithelial (MCF-10F) and human breast cancer (MCF-7) cell lines.

Cell line	D.T. (h)	S.D. ($x10^6$)	P.E. (%)	G0/G1 (%)#	S (%)#	G2/M (%)#	A.I.G. (%)
MCF10F-PV#1	29.4	2.9	6.2	60.5	18.8	20.7	0
MCF10F-PKIP#1	32.6	1.2	2.1	66.6	14.4	19.0	0
MCF10F-PKIP#2	34.4	0.3	0.6	71.8	10.7	19.5	0
MCF7-PV#1	28.4	4.2	33	41.2	35.9	22.9	17
MCF7-PV#2	27.2	4.8	27	34.6	43.3	22.1	17
MCF7-PV#3	29.2	4.3	26	48.6	34.8	16.5	18
MCF7-PKIP#9	35.9	3.6	21	50.5	35.7	13.8	11
MCF7-PKIP#14	37.9	3.6	10	59.5	29.1	11.4	8
MCF7-PKIP#23	37.2	3.5	9	60.2	24.6	15.2	5
MCF7-PKIP#45	39.6	2.4	7	62.4	25.1	12.5	1

The data reported are the results of a typical experiment for each cell line. Values are the means of triplicate determinations. Standard deviations for individual assays were less than 25% of the mean. Similar results were obtained in replicate experiments.

D.T. = Doubling time, in hours (h), corresponds to the initial exponential phase of cell growth.

S.D. = Saturation density, represents the total number of cells per 35-mm well when the cultures reached a plateau in their growth.

P.E. = Plating efficiency.

A.I.G. = Anchorage independent growth, expressed as colony forming efficiency in soft agar.

Exponentially growing cultures of the indicated cell lines were analyzed by flow cytometry. The values represent the percentage of the total cell population in each phase of the cell cycle.

For additional details see Materials and Methods in ref. 18.

(7) CONCLUSIONS FOR FINAL REPORT

The overall purpose of this project was to examine the hypothesis that abnormalities in the expression of cyclin D1 and related cell cycle control genes play an important role in the multistage process of breast carcinogenesis. The results we have obtained fully confirm this hypothesis and also suggest new strategies for breast cancer chemoprevention and therapy. Our results also revealed the existence of complex homeostatic feedback mechanisms that regulate the levels of cell cycle control proteins in breast cancer cells.

In studies carried out in 1994 and 1995 we demonstrated for the first time that carcinogen-induced rat mammary tumors display abnormalities in the expression of several cell cycle-related genes, especially cyclins D1 and E. Since similar changes are seen in human breast carcinomas the rat system may be a useful model for studying the role of these abnormalities in breast cancer causation, prevention and treatment. In a second set of studies we found that stable overexpression of an exogenous cyclin D1 cDNA in the HBL-100 human mammary epithelial cell line markedly inhibits rather than enhances the growth of these cells. This effect is not confined to the HBL-100 cells since we found that overexpression of cyclin D1 also inhibits the growth of another human mammary epithelial cell line Hs578Bst and the mouse mammary epithelial cell line HC11. These findings are in contrast to our previous results in which stable overexpression of cyclin D1 in rat fibroblasts enhanced growth and tumorigenicity. Thus, the effects of increased expression of cyclin D1 on cell growth are a function of the context of particular cell types, presumably reflecting the levels of expression of other cellular genes. Our results are consistent with evidence that the level of expression of cyclin D1 in primary human breast cancers cannot in itself be used as a marker of cell proliferation or prognosis.

In studies carried out in 1995, 1996 and 1997 we obtained evidence that overexpression of cyclin D1 in breast cancer cell lines can induce the cell cycle inhibitory protein $p27^{Kip1}$, and enhance apoptosis. We found that cyclin E, which also plays a critical role in the G1 to S progression of the cell cycle, is also frequently overexpressed in breast cancer cell lines. We found that ectopic overexpression of cyclin E stimulates the growth of fibroblast cell lines but inhibits the growth of some mammary epithelial cell lines. The latter effects are associated with increased expression of $p27^{Kip1}$. These findings, together with our previous results, provide evidence for the existence of homeostatic feed-back loops between cyclin D1 and $p27^{Kip1}$ and between cyclin E and $p27^{Kip1}$. This may explain the otherwise paradoxical finding that human breast cancer cell lines and primary tumors often express relatively high levels of $p27^{Kip1}$ even though this protein is an inhibitor of cell cycle progression. We found that overexpression of cyclin E in mammary epithelial cells was associated with increased resistance to growth inhibition by TGF-β. This effect, together with other effects of cyclin E, cyclin D1 and $p27^{Kip1}$, may contribute to mammary tumorigenesis. In a study of 168 primary human breast cancers we found increased expression of cyclin D1 in 68% of these cases. Therefore, overexpression of cyclin D1 is one of the most frequent molecular defects in human breast cancer.

In studies carried out in 1997 and 1998 we obtained further evidence for an aberrant increase in the expression of $p27^{Kip1}$ in human breast cancer cell lines and in a subset of primary human breast cancers. We also found dysregulation of $p27^{Kip1}$ during the cell cycle human breast cancer cell lines. A possible explanation for the paradoxical increase in $p27^{Kip1}$ in the breast

cancer cell lines was that they had become refractory to the inhibitory effects of this protein. To address this question, in a very recent study we transfected the MCF7 breast cancer cell line and the MCF10F non-tumorigenic mammary epithelial cell line with a vector containing the $p27^{Kip1}$ cDNA to obtain derivatives that express increased levels of $p27^{Kip1}$. The increased expression of $p27^{Kip1}$ in both of these cell lines was associated with lengthening of the G1 phase, an increase in the doubling time, a decreased saturation density and a decreased plating efficiency. In the MCF7 cells anchorage-independent growth and *in vivo* tumorigenicity were also suppressed. These effects were associated with decreased cyclin E - associated kinase activity, in both cell lines. The increased expression of $p27^{Kip1}$ was associated with a decreased level of expression of cyclin D1 in the MCF10F cells but an increased level of the cyclin E protein. Thus, breast cancer cells are still responsive to $p27^{Kip1}$ mediated inhibition of cell growth despite the high basal level of this protein. These results suggest that therapeutic strategies that further increase the level of expression of $p27^{Kip1}$, or mimic its activity, might be useful in breast cancer chemoprevention or therapy.

This grant has resulted in 7 peer reviewed publications and 3 review articles (see Appendix for reprints).

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Note that reference numbers 6, 9, 11, 15, 16, 17 and 18 are publications supported by this grant.

(9) APPENDIX

Reprints of publications supported by this grant.

(11) a) BIBLIOGRAPHY OF ALL PUBLICATIONS ON THIS GRANT

1. Han, E. K-H., Sgambato, A., Jiang, W., Zhang, Y-J., Santella, R.M., Doki, Y., Cacace, A., Schieren, I., and Weinstein, I.B. Stable overexpression of cyclin D1 in a human mammary epithelial cell line prolongs the S-phase and inhibits growth. Oncogene, 10, 953-961, 1995.

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b) PERSONNEL WHO RECEIVED PAYMENT ON THIS GRANT

E.K.-H. Han
A. Sgambato
W.-Q. Xing

Stable overexpression of cyclin D1 in a human mammary epithelial cell line prolongs the S-phase and inhibits growth

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Amplification and/or increased expression of cyclin D1 occurs in an appreciable fraction of primary human breast carcinomas and several other types of human cancer. In addition, overexpression of cyclin D1 in rodent fibroblasts enhances growth and malignant transformation. The present study demonstrates that the extent of amplification and expression of cyclin D1 varies widely amongst a series of cell lines established from normal human mammary epithelium or human breast carcinomas. The HBL-100 mammary epithelial cell line did not display amplification or increased expression of cyclin D1. We used retrovirus-mediated transduction to obtain derivatives of this cell line that stably expressed relatively high levels of an exogenous cyclin D1 cDNA. These derivatives displayed an increased doubling time, decreased saturation density, decreased cloning efficiency, decreased anchorage-independent growth, an increased fraction of cells in the S-phase, and decreased tumorigenicity. Thus, increased expression of cyclin D1 in this cell line markedly inhibits rather than enhances growth, which may be due to the prolongation of S-phase.

Keywords: cyclins; cell cycle; breast cancer

Introduction

There is now abundant evidence that, as originally discovered in lower organisms, the orderly progression of dividing mammalian cells through the various phases of the cell cycle is governed by a series of proteins called cyclins which exert their effects through specific cyclin-dependent protein kinases. Because of the central role of these genes in cell cycle control it seems likely that disturbances in the functions of these genes play a role in tumorigenesis (For review see Pardee, 1989; Nurse, 1990; Pines, 1991; Sherr, 1993). At least eight cyclin genes have been identified in mammalian cells. They are classified into three major groups: G1 cyclins, A cyclins and B cyclins. The G1 cyclins (C,D1–3 and E) are maximally expressed during G1 and presumably regulate transition of the cell cycle from G1 into the S-phase (Lew *et al.*, 1991; Motokura *et al.*, 1991; Matushime *et al.*, 1991; Dulic *et al.*, 1992; Koff *et al.*, 1992). Cyclin A is highly expressed in early S-phase of the cell cycle and presumably enhances transition through the S-phase. Two B-type cyclins (B1 and B2) are apparently important for the cell entry into and exit from mitosis. Cyclins act by binding to and stimulating the activities of a series of cyclin-dependent protein kinases (CDK). The activities of these CDKs is further modulated by protein phosphorylation and de-phosphorylation (Hunter and Pines, 1991; van den Heuvel & Harlow, 1993; Kato *et al.*, 1994), and by a group of specific inhibitory proteins. To date, at least six mammalian CDKs, (1-6) have been identified (Hunter, 1993; Sherr, 1993; Meyerson & Harlow *et al.*, 1994). CDK1 (also called Cdc2) is involved in regulation of the G2/M transition, in association with cyclin B (Pines and Hunter, 1989). Cyclin A can also associate with CDK1 and apparently this complex also plays a role in the G2/M transition (Pagano *et al.*, 1992). CDK2 is involved in regulating the G1/S transition by its association with cyclin E (Pagano *et al.*, 1992; Ohtsubo and Roberts, 1993; Tsai *et al.*, 1993) and cyclin A (Girard *et al.*, 1991). CDK4 is the major catalytic partner for cyclin D1, and cyclins D2 and D3 (Ewen *et al.*, 1993), and this complex can phosphory-late the retinoblastoma protein (pRB) (Kato *et al.*, 1993). Cyclin D1 also complexes with CDK5 (Xiong *et al.*, 1992) and CDK6 (Meyerson & Harlow, 1994), but the function of these complexes is not known. It can also complex with the DNA replication factor, proliferating cell nuclear antigen (PCNA) (Xiong *et al.*, 1992) and the retinoblastoma protein (pRB) (Dowdy *et al.*, 1993). Several studies indicate that cyclin D1 is involved in inactivating the function of pRB, presumably through phosphorylation and/or the formation of a physical complex (Hinds *et al.*, 1992; Dowdy *et al.*, 1993; Kato *et al.*, 1993). Furthermore, cyclin D1 expression is positively regulated by pRB (Muller *et al.*, 1994; Lukas *et al.*, 1994b), thus providing an autoregulatory feedback loop.

Recently, several proteins that inhibit activities of CDKs have been identified (for review see Peters, 1994). The protein p21 (WAF1/CIP1), whose synthesis is induced via the p53 protein in response to DNA damage, binds to various cyclin-CDK complexes (CDK2-cyclin A, cyclin E, and CDK4-cyclin D1) and inhibits their activation (Harper *et al.*, 1993; Xiong *et al.*, 1993; El-Deiry *et al.*, 1994), thus causing cell cycle arrest. Similarly, the protein p27 binds to the cyclin D1-CDK4 and cyclin E-CDK2 complexes and inactivates their function, thus arresting cells at G1/S (Polyak *et al.*, 1994a; Toyoshima & Hunter, 1994). This occurs

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when cells undergo contact-dependent inhibition of growth or inhibition of growth in response to treatment with TGF-β (Polyak *et al.*, 1994b) . Recent studies indicate that a protein designated p15 mediates the effect of TGF-β (Hannon & Beach, 1994). The protein p16 (INK4/MTS1) binds to and inhibits the activity of CDK4. This protein is mutated or deleted in a variety of human tumor cell lines and certain primary tumors (Serrano *et al.*, 1993; Kamb *et al.*, 1994).

There is increasing evidence that several types of human tumors display abnormalities in cyclin genes (for review see Motokura & Arnold, 1993a, b). One of the earliest examples was the finding that the cyclin A gene was the site of integration of the hepatitis B virus in a human hepatocellular carcinoma (Wang *et al.*, 1990). This proviral integration into the cyclin A gene locus resulted in the synthesis of a stable HBV-cyclin A fusion protein (Wang *et al.*, 1992). However, this appears to be a rare event in human hepatocellular carcinomas. The cyclin E gene is often deregulated and overexpressed in human breast tumor cell lines and in primary human breast, colon and prostate carcinomas (Buckley *et al.*, 1993; Keyomarsi and Pardee, 1993), but, with rare exceptions, it is not usually amplified (Keyomarsi and Pardee, 1993). There are numerous examples of abnormalities in the cyclin D1 gene. This gene, also termed prad 1 or bcl-1, is located at chromosome 11q13. Chromosomal rearrangements at this locus in parathyroid tumors (Motokura *et al.*, 1991) or certain B cell lymphomas (Rosenberg *et al.*, 1991; Withers *et al.*, 1991) leads to increased expression of this gene. More importantly, the cyclin D1 is amplified and overexpressed in a significant fraction of primary human breast carcinomas (Lammie *et al.*, 1991; Schuuring *et al.*, 1992), esophageal carcinomas (Jiang *et al.*, 1992, 1993a) and several other types of tumors (Berenson *et al.*, 1990; Proctor *et al.*, 1991; Zhang *et al.*, 1993; Schauer *et al.*, 1994), both in cell lines and in primary tumors. Recent studies indicate that stable overexpression of cyclin D1 in rodent fibroblasts enhances their tumorigenicity in nude mice (Jiang *et al.*, 1993b) and that cyclin D1 cooperates with a defective adenovirus E1A (Hinds *et al.*, 1994) or an activated *ras* oncogene (Lovec *et al.*, 1994) in the transformation of rodent fibroblasts. Furthermore, overexpression of cyclin D1 in transgenic mice results in mammary hyperplasia and tumors of the mammary epithelium (Wang *et al.*, 1994). Thus, overexpression of cyclin D1 could play a critical role in tumor formation in humans. In the present study we have examined in detail the extent of amplification and overexpression (at both the mRNA and protein levels) of cyclin D1 in a series of cell lines originally established from apparently normal human mammary epithelium or human breast carcinomas. Since we found that the HBL-100 mammary epithelial cell line did not display amplification or detectable overexpression of this gene we developed derivatives of this cell line that stably express relatively high levels of an exogenous cyclin D1 cDNA. To our surprise we found that this markedly inhibited, rather than stimulated, the growth of these cells, and also prolonged the duration of their S-phase. The

Results

Amplification and increased expression of the endogenous cyclin D1 gene in a series of human mammary epithelial and breast carcinoma cell lines

Several laboratories have studied amplification and expression of cyclin D1 in mammary tumors and cell lines (Lammie *et al.*, 1991; Schuuring *et al.*, 1992; Buckley *et al.*, 1993; Gillett *et al.*, 1994). However, only two studies have investigated cyclin D1 expression at the protein level (Bartkova *et al.*, 1994; Lukas *et al.*, 1994a) and there have been no previous studies on the growth characteristics of mammary epithelial cells engineered to stably overexpress an exogenous cyclin D1 sequence. Therefore, in our initial studies we examined in parallel cyclin D1 amplification and expression, at both the mRNA and protein levels, in two human epithelial cell cultures originally established from normal human mammary epithelium and in five cell lines originally established from human breast carcinomas. Genomic DNAs were isolated, and digested with either EcoRI or HindIII and then analysed for amplification of cyclin D1 by southern blot analysis. Equal amounts of DNA were loaded onto the membranes as determined by ethidium bromide staining of the membranes (data not shown). As shown in Figures 1A and 1B, cyclin D1 was highly amplified (about 10-fold) in the MDA-MB-134 and MDA-MB-330 carcinoma cell lines, when compared to the normal mammary epithelial cell line Hs578Bst. A moderate level of cyclin D1 amplification (about 2- to 3-fold) was seen in the MCF-7, ZR-75-1 and T-47D breast carcinoma cell lines. The HBL-100 cell line, which was originally established from apparently normal human mammary epithelium (Gaffney, 1982) but expresses SV40 large T antigen and is partially transformed (Caron de Fromentel *et al.*, 1985), did not show amplification of cyclin D1. These results are, in general in agreement with previously published reports on breast cancer cell lines (Lammie *et al.*, 1991; Schuuring *et al.*, 1992). Northern blot analyses (Figure 2A) indicated a very high level of cyclin D1 mRNA in the MDA-MB-134 cells, a moderately high level in the Hs578Bst, MCF-7, and ZR-75-1 cells, a somewhat lower level in the T-47D cells and an extremely low level in the HBL-100 cells. The MDA-MB-330 cell line was not examined in this study. The ethidium bromide staining which was done to control for RNA loading (Figure 2B) suggested that the relative level of cyclin D1 mRNA in MDA-MB-134 cells is even higher than that revealed in Figure 2A. Western blot analysis (Figure 1C) indicated a very high level of cyclin D1 protein in the MDA-MB-134 cells, which also displayed the highest level of cyclin D1 amplification and mRNA expression (Figures 1A and 2A). A moderate level of cyclin D1 protein was present in the MDA-MB-330 cells, a low level in the ZR-75-1 and MCF-7 cells and trace or undectable level in the T-47D carcinoma cell line. The Hs578Bst and HBL-100 cells originally established from normal mammary epithelial cells also displayed only trace amounts of cyclin D1 protein (Figure 1C). Thus in this series of cell lines there was a general, but not uniform, correlation between cyclin D1 gene amplification and

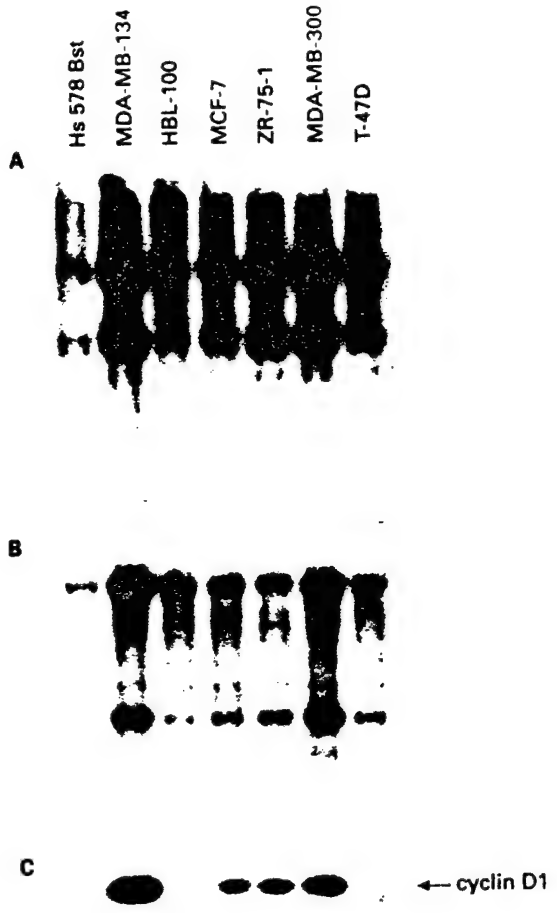

Figure 1 (A) and (B) Southern blot analysis of cyclin D1 in human mammary epithelial and breast carcinoma cell lines. Genomic DNA (5 μg each) was isolated from each of the indicated cell lines and digested with either EcoRI (A) or HindIII (B). Digested samples were electrophoresed, transferred to a membrane and hybridized with a ^{32}P-labelled cyclin D1 probe. (C) Western blot analysis of protein extracts from the same cell lines using a cyclin D1 antibody. For additional details see Materials and methods

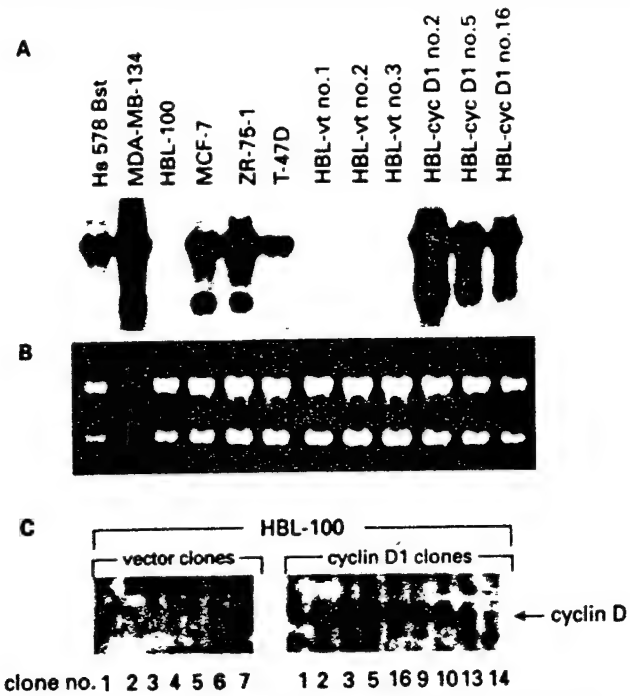

Figure 2 (A) Northern blot analysis of cyclin D1 mRNA. Total RNAs were isolated from the indicated cell lines and derivatives of the HBL-100 cell line. Samples (10 μg each) were electrophoresed, transferred to a membrane and hybridized with a ^{32}P-labelled human cyclin D1 cDNA probe. Three vector control clones (HBL-vt no. 1, no. 2 and no. 3) and three cyclin D1 overexpressor clones (HBL-cyc D1 no. 2, no. 5 and no. 16) are also shown. (B) Ethidium bromide staining of the membrane is shown as a control for RNA loading. (C) Western blot analysis showing the levels of expression of cyclin D1 protein in a series of HBL-100 vector control and cyclin D1 overexpressing clones. For additional details see Materials and methods

(compare Figures 1 and 2). T-47D cells displayed a moderate level of amplification of cyclin D1 and mRNA expression but only a very low level of cyclin D1 protein. Also, neither Hs578Bst not HBL-100 cells displayed evidence of cyclin D1 amplification, and yet Hs578Bst cells displayed a higher level of cyclin D1 mRNA that HBL-100 cells (Figures 1A and 2A). Presumably, these apparent discrepancies reflect differences in the regulation of cyclin D1 expression at the levels of mRNA transcription or stability and protein translation or stability (see also Discussion). Previous studies indicate that cells that do not express the Rb protein also express low levels of cyclin Di (Jiang *et al.*, 1993a; Muller *et al.*, 1994). During the course of these studies other investigators reported that the level of cyclin D1 protein expression in primary human breast carcinomas does not always correlate with the extent of gene amplification (Buckley *et al.*, 1993; Gillett *et al.*, 1994).

Development of derivatives of the HBL-100 cell line that stably express high levels of cyclin D1

The above study indicated that the cyclin D1 gene was neither amplified nor expressed at significant levels, at both the mRNA and protein levels, in the HBL-100 cell line. Thus, we decided to stably overexpress an exogenous cyclin D1 cDNA in this cell line by using a retrovirus-derived vector, to determine possible effects on growth, cell cycle parameters and gene expression. In previous studies we used the same retrovirus derived vector (pMV7-CCND1) to stably overexpress cyclin D1 in rat R6 fibroblasts (Jiang *et al.*, 1993b). Following transduction and selection for neomycin resistance (see Materials and methods) a number of vector control and cyclin D1 clones were obtained. As shown in Figure 2C, cyclin D1 protein was not detected by Western blot analysis of proteins extracted from seven neomycin resistant vector control clones. However, appreciable but varying amounts of cyclin D1 protein were detected in nine neomycin resistant clones derived from HBL-100 cells transduced with the PMV7-CCND1 vector (Figure 2C). We chose three vector control clones (HBL-vt no. 1, HBL-vt no. 2, HBL-vt no. 3) and three cyclin D1 clones (HBL-cycD1 no. 2, HBL-cycD1 no. 5, HBL-cycD1 no. 16) for further studies. Densitometric analysis of western blots indicated that the HBL-cycD1 no. 2 clone expressed about a 7-fold increase in cyclin D1 protein, and the HBL-cycD1 no. 5 and no. 16 clones expressed about a 5-fold increase, when compared to the vector control clones. Immunofluorescence analysis of intact cells using a cyclin D1 specific antibody confirmed the high level of expression, and also demonstrated nuclear localization, of the cyclin D1 protein in the latter three clones (data not shown). Northern blot analysis demonstrated a high level of cyclin D1 mRNA, of the

expected size (4.5 kb), in these three clones, especially clone no. 2, but cyclin D1 mRNA was not detected in the three vector control clones (Figure 2A).

Alterations in cell growth in the cyclin D1 overexpressing HBL-100 clones

To examine the growth properties of the cyclin D1 overexpressor clones, several parameters were studied including exponential doubling time, saturation density, anchorage-independent growth, and tumorigenicity in nude mice (see Table 1). When grown in 10% FCS the parental HBL-100 cells and the three vector clones showed similar doubling times (about 28–29 h). The doubling times of the three cyclin D1 overexpressor clones were increased to about 34–36 h. It is of interest that similar values were obtained when the vector control or overexpressor clones were grown in the presence of 1% FCS (Table 1). Thus HBL-100 cells have a relatively low requirement for serum and this is also true of the cyclin D1 overexpressor clones. The saturation density of the three cyclin D1 clones was also reduced when compared to the vector control clones. The decrease was greatest with overexpressor clone no. 2 which had the highest expression of cyclin D1 (Table 1 and Figure 1C). The cloning efficiencies of the three overexpressor clones in monolayer culture were also markedly reduced when compared to the HBL-100 parental and three vector control cell lines (Table 1).

Consistent with previous reports (Caron de Fromental *et al.*, 1985; Saint-Ruf *et al.*, 1988), the HBL-100 parental cells and the vector control derivatives were able to grow in soft-agar. The three overexpressor clones showed a decreased cloning efficiency in agar when compared to the control cells (Table 1). In addition, the colonies formed by the cyclin D1 overexpressor clones were flatter and more monolayer-like than those formed by the vector control cells (data not shown). When these cell lines were tested for tumor formation in nude mice, none of the cyclin D1 overexpressor clones formed tumors, whereas the parental HBL-100 cell line and one of the three vector control clones (HBL-vt no. 3) did form tumors (Table 1).

The above studies clearly indicate that an approximately 5–7-fold increased expression of the cyclin D1 protein in HBL-100 cells markedly inhibited the growth of HBL-100 cells, by several parameters and decreased the tumorigenicity of these cells. A possible explanation is that overexpression of cyclin D1 in this cell line increases the production of an extracellular growth inhibitory substance. It is known that the parental HBL-100 cells do secrete a basic fibroblast growth factor (Souttou *et al.*, 1994). In preliminary experiments, however, we have been unable to demonstrate that conditioned medium obtained from the HBL-100 cyclin D1 overexpressor clones inhibits the growth of the parental HBL-100 cells (unpublished studies).

Effects of cyclin D1 overexpression on cell cycle parameters

Because there is increasing evidence that in other cell systems cyclin D1 plays a critical role in cell cycle progression, especially in the mid-to late G1 phase (Jiang *et al.*, 1993b; Quelle *et al.*, 1993; Resnitzky *et al.*, 1994), it was of interest to examine HBL-100 control and cyclin D1 overexpressor cells obtained from exponentially dividing non-synchronized cultures by flow cytometry after 'tagging' the DNA of these cells with propidium iodide. Representative flow cytometry profiles for the vector control cell line HBL-vt no. 1 and for the cyclin D1 overexpressor cell line HBL-cycD1 no. 2 are shown in Figure 3 and the data obtained on a larger series of cells tabulated in Table 2. It is apparent that overexpression of cyclin D1 decreases the percent of the total cell population that is in the G0/G1 phase, increases the percent that is in S-phase, and has no consistent effect on the percent in the G2/M phase. Although there are some variations between the individual control and overexpressor clones (Table 2), this general pattern was also seen in additional experiments (data not shown). Since the cyclin D1 overexpressor clones have a longer exponential doubling time (Table 1) than the control cells we also used the flow cytometry data and the exponential doubling times to calculate the approximate length in hours of each of these three phases of the cell cycle in the various cell lines (Table 2). It can be seen that the length of the G0/G1 phase for both the control and cyclin D1 overexpressors is about 15 h and that the length for the G2/M phase for both cell types is about 5 h. On the other hand the length of the S-phase for the three cyclin D1 overexpressor clones tended to be longer than that for the control clones, about 11–16 h *vs* about 7–9 h, respectively. Thus, the increased doubling time of the cyclin D1 overexpressor clones appears to be due, mainly, to a lengthening of the duration of the S-phase. It is of interest that the MDA-MB-134 cell line which displays amplification and high expression of the endogenous cyclin D1 gene (Figure 1) also grows slowly. Cell cycle analysis of

Table 1 Growth properties of cyclin D1 overexpressing HBL-100 cells*

	Growth in monolayer culture						
	Doubling time(h)		*Saturation density($\times 10^5$)*		*Cloning*	*Growth in agar*	*Tumorigenicity*
Cell line	*1%FCS*	*10%FCS*	*1%FCS*	*10%FCS*	*Efficiency (%)*	*Efficiency (%)*	*in nude mice*
HBL-100	29.8	28.8	3.2	3.1	13.0	9.6	2/2
HBL-vt no.1	28.8	28.8	3.0	3.0	17.6	8.4	0/2
HBL-vt no.2	28.5	27.6	3.2	3.0	18.0	7.2	0/2
HBL-vt no.3	29.4	28.8	3.2	3.4	26.0	6.5	2/2
HBL-cycD1 no.2	36.8	36.0	0.5	0.9	7.1	5.0	0/2
HBL-cycD1 no.5	34.1	33.6	1.5	2.0	5.2	4.1	0/2
HBL-cycD1 no.16	34.9	33.6	1.5	2.3	9.2	5.3	0/2

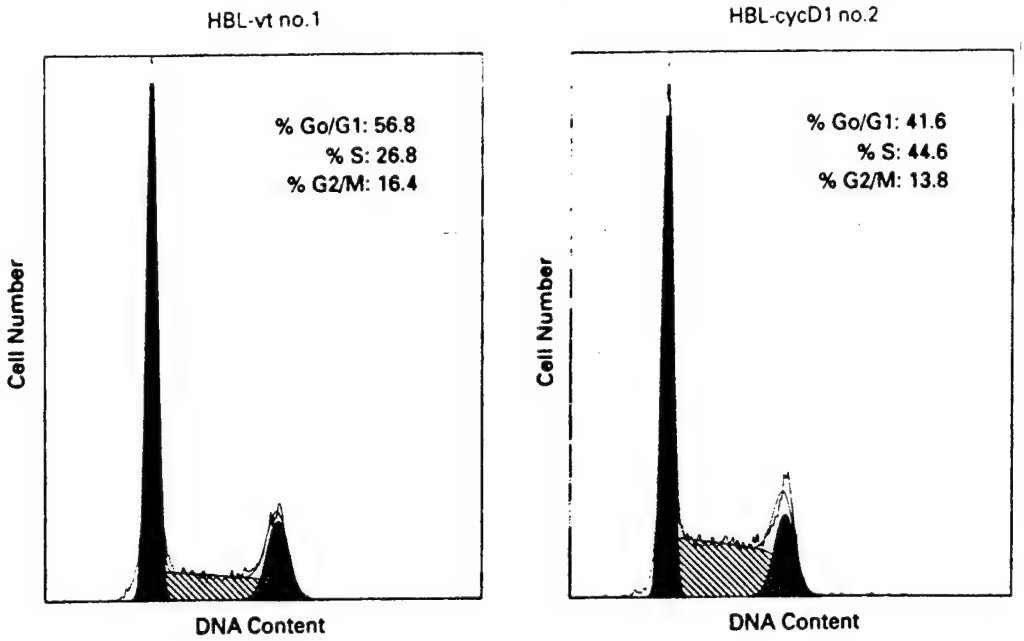

Figure 3 Flow cytometry profiles of HBL-vt no. 1 and HBL-cycD1 no. 2 clones. For additional details see Materials and methods

Table 2 Flow cytometric analysis of cyclin D1 overexpressing derivatives of HBL-100 cells*

	G0/G1		S		G2/M	
Cell line	*%cells*[a]	*Time(h)*[b]	*%cells*[a]	*Time(h)*[b]	*%cells*[a]	*Time(h)*[b]
HBL-100	50.2	14.5	30.6	8.8	19.2	5.5
HBL-vt no.1	56.8	16.4	26.8	7.7	16.4	4.7
HBL-vt no.2	57.7	15.9	24.9	6.9	17.4	4.8
HBL-vt no.3	59.1	17.0	26.2	7.5	14.7	4.3
HBL-cycD1 no.2	41.6	15.0	44.6	16.1	13.8	4.9
HBL-cycD1 no.5	41.6	14.0	44.6	15.0	13.8	4.6
HBL-cycD1 no.16	47.5	16.0	32.4	10.9	20.1	6.7

*Exponentially growing cultures of the indicated cell lines were collected and their DNA content analysed by flow cytometry. For additional details see Materials and methods. [a]The values indicate % of the total cell poulation in the indicated phase of the cell cycle. [b]The values indicate the length of each phase of the cell cycle based on the doubling time of the indicated cell line

exponentially dividing cultures indicated the following distribution: G0/G1, 74%; S, 17%; and G2/M, 9% (data not shown). These values are similar to those obtained by Tam *et al.* (1994). Thus, the slow growth of these cells appears to be due mainly to a lengthening of the G1-phase of the cell cycle rather than the S-phase. Presumably this difference between the effects of high levels of expression of cyclin D1 on cell cycle progression in the HBL-100 and MDA-MB-134 cell lines is due to differences between these cell line in the expression of other genes (see Discussion), but this aspect requires further study.

Effect of cyclin D1 overexpression on the expression of other critical genes

In view of the above described effects on growth control and cell cycle parameters we examined the vector control and cyclin D1 overexpressor HBL-100 clones for expression at the mRNA level of two genes that play a critical role in growth control, c-*myc* and c-*jun*, and on the gene cyclin A which is specifically implicated in control of the S-phase of the cell cycle. In this study we also included several other cell lines originally studied for cyclin D1 amplification and expression in Figures 1 and 2. Although there were some variations, northern blot analyses revealed that the three cyclin D1 overexpressors clones do not display any consistent differences with respect to levels of the c-*myc*, c-*jun*, or cyclin A mRNAs, when compared to the parental HBL-100 or vector control clones (Figure 4). Nor does there appear to be a consistent correlation between the extent of amplification or expression of the endogenous cyclin D1 in the other six cell lines (Figures 1 and 2) and the level of expression of c-*myc*, c-*jun* and cyclin A (Figure 4). Thus it would appear that the decreased growth potential seen in the HBL-100 cell can not be simply attributed to decreased expression of immediate early response genes involved in mitogenesis. Nor does the apparent prolongation in S-phase in the overexpressor cells simply due to decreased expression of cyclin A. Numerous other factors remain, of course, to be examined.

Discussion

The present studies demonstrate that a series of five human breast carcinoma cell lines differ markedly with respect to the extent of amplification of the cyclin D1 gene. In general amplification of this gene was

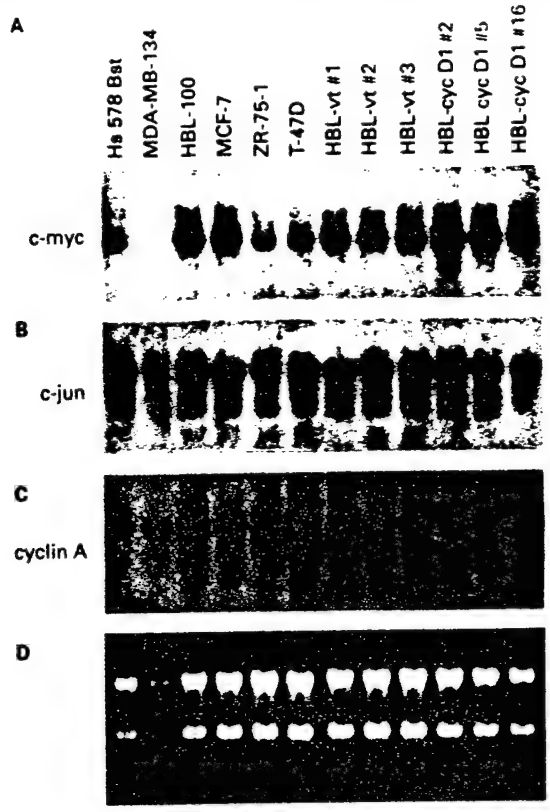

Figure 4 Northern blot analysis of the level of expression of mRNAs for c-*myc* (A), c-*jun* (B) and cyclin A (C). Total RNAs were isolated from the indicated cell lines, electrophoresed and hybridized to the indicated ^{32}P-labelled probes. (D) Ethidium bromide staining of the membranes is shown as a control for RNA loading. For additional details see Materials and methods

associated with increased expression at the protein level but amongst these cell lines there was not a simple correlation between the extent of cyclin D1 gene amplification and expression. Two cell lines, Hs578Bst and HBL-100, originally established from normal human mammary epithelium, did not show amplification of the cyclin D1 gene and expressed only a very low level of the corresponding protein despite prolonged serial passage *in vitro*. These findings are consistent with other recent studies on cyclin D1 amplification and expression in primary human breast carcinomas and cell lines (Buckley *et al.*, 1993; Lukas *et al.*, 1994a).

The most important new finding in the present study is that stable overexpression of an exogenous cyclin D1 cDNA in the HBL-100 cell line markedly inhibits rather than enhances the growth of these cells. This effect is not confined to the HBL-100 cells since we have found that overexpression of cyclin D1 also inhibits the growth of Hs578Bst cells (unpublished studies). Furthermore, as mentioned in the Results section, the MDA-MB-134 cells which display amplification and high expression of the endogenous cyclin D1 gene also grow slowly. Nevertheless, further studies are required to determine to what extent our findings can be generalized to other mammary epithelial and breast carcinoma cell lines. Our findings with HBL-100 and Hs578Bst cell lines are in contrast to previous results obtained in this laboratory, using the same cyclin D1 cDNA retrovirus-derived construct, in which stable overexpression of cyclin D1 in the rat R6 fibroblast cell line enhanced the growth of these cells and increased their tumorigenecity in nude mice (Jiang *et al.*, 1993b). Other investigators have found that cyclin D1 can cooperate with a defective adenovirus E1A gene (Hinds *et al.*, 1994) or an activated *ras* oncogene (Lovec *et al.*, 1994) in enhancing the transformation of murine fibroblasts. At the present time it is not clear why stable overexpression of cyclin D1 should have reciprocal effects on growth in certain human mammary epithelial and rodent fibroblast cell lines. There are, however, other similar examples. Thus, TGF-β often stimulates the growth of fibroblasts but inhibits the growth of epithelial cells (Moses *et al.*, 1990) and protein kinase C-β1 (PKC-β1) stimulates the growth of rat fibroblasts (Housey *et al.*, 1988) but inhibits the growth of human colon cancer cells (Choi *et al.*, 1990).

Nevertheless, the findings in the present study are still surprising since about 20–30% of primary human breast tumors display amplification and increased expression of cyclin D1 and amplification of this locus in breast cancer has been associated with a poorer prognosis (Schuuring *et al.*, 1992). A possible explanation is that a moderate increase in the expression of cyclin D1 can enhance cell growth but a high level of expression can have an inhibitory effect, even in the same cell type. Indeed, it is of interest that the breast carcinoma cell lines MDA-MB-134 and MDA-MB-330, that have very high amplification and expression of the endogenous cyclin D1 gene (Figures 1 and 2), grow relatively slowly in cell culture when compared to the breast carcinoma cell lines that express a moderate level of cyclin D1 (unpublished studies). Studies utilizing anti-sense cyclin D1 are in progress to determine if the relatively slow growth of the former cell lines is specifically due to the high level of expression of cyclin D1 in these cell lines. An alternative possibility relates to the fact that in some human breast tumors the amplified region at 11q13 contains genes in addition to cyclin D1, that are amplified and overexpressed (Fantl *et al.*, 1993). It is possible that these gene products stimulate the growth of breast carcinomas and that they rather than cyclin D1 are responsible for the reported poorer prognosis of patients with breast tumors that display amplification of the 11q13 region (Schuuring *et al.*, 1992). We should also emphasize that the effects of stable expression of an exogenous cyclin D1 cDNA in HBL-100 cells might be due not only to the high level of cyclin D1 produced in these cells but also to the fact that the expression of the exogenous form may not be subject to the same cell cycle regulation as the endogenous form. Nevertheless it is possible that in same tumor cells there is a similar dysregulation of expression of the endogenous cyclin D1 gene.

The most likely explanation, however, for the present results is that the effects of increased expression of cyclin D1 on the growth of a given cell line depends on the level of expression of other genes in that cell line. Thus, there are examples where ectopic expression of cyclin D1 can be toxic to even fibroblasts (Quelle *et al.*, 1993; Pagano *et al.*, 1994). Possible factors that might affect this balance and its biologic consequences include the level of expression and/or the consequence of mutations in the Rb gene, specific

CDKs, or the inhibitory proteins p16, p21 or p27. The Rb protein is apparently functionally inactive in HBL-100 cells, since they express the SV40 T antigen (Bartkova *et al.*, 1994; Tam *et al.*, 1994). It is unlikely, however, that this is the critical factor since, as mentioned above, in unpublished studies we have found that overexpression of cyclin D1 in Hs578Bst cells, which do not have the SV40 T antigen, also inhibits cell growth.

As discussed in the Introduction it appears that cyclin D1 often enhances the progression of cells through the G1 phase mainly by inhibiting the function of pRb. Indeed introduction of anti-cyclin D1 antibodies fails to inhibit the entry into S-phase in cells that are functionally Rb negative, including HBL-100 cells (Bartkova *et al.*, 1994; Lukas *et al.*, 1994b). Therefore, it would appear that the growth inhibitory effects of cyclin D1 overexpression in HBL-100 cells are exerted via proteins other than the Rb protein. A critical factor might be the PCNA protein since this protein can complex with cyclin D1 and CDK4 (Xiong *et al.*, 1992), and at the same time PCNA plays an essential role in DNA replication and repair (Pagano *et al.*, 1994). Indeed, Pagano *et al.* (1994) found that acute overexpression of cyclin D1 in fibroblasts prevented them from entering S-phase and this effect was abolished by coexpression of cyclin D1 with PCNA. Our hypothesis is consistent with recent evidence indicating that increased expression of cyclin D1 can inhibit DNA repair synthesis presumably by tying up the PCNA protein (Pagano *et al.*, 1994). An inhibitory effect on PCNA function might also explain why overexpression of cyclin D1 increases the doubling time of HBL-100 cells, as discussed below.

Several recent studies indicate that overexpression of cyclin D1 in fibroblasts, shortens the G1-phase but lengthens the S and G2/M phases (Jiang *et al.*, 1993b; Quelle *et al.*, 1993; Resnitzky *et al.*, 1994). One report indicated that overexpression of cyclin D1 resulted in a shorter doubling time (Quelle *et al.*, 1993) relative to control cells but others reported no change in the doubling time (Jiang *et al.*, 1993b; Resnitzky *et al.*, 1994). In a recent study, induced expression of cyclin D1 in synchronized T-47D breast cancer cells, employing a zinc-responsive metallothionen promoter resulted in a shorter G1 phase but had no effect on the duration of the S-phase (Musgrove *et al.*, 1994). In the present studies, however, stable overexpression of cyclin D1 in HBL-100 cells increased their doubling time (Table 1). This appeared to be due mainly to a lengthening of the duration of the S-phase from about 3 to 9 h, since there was no appreciable change in the duration of the G1 and G2/M phases when compared to control cells (Table 2). HBL-cycD1 no. 2 cells which expressed the highest level of cyclin D1 protein (Figure 1) displayed the longest doubling time and length of the S-phase (Tables 1 and 2).

To explain the above findings we propose that the increased expression of cyclin D1 in HBL-100 cells does not shorten the duration of G1 since the cells are already functionally Rb minus, as discussed above. At the same time, we propose that high levels of the cyclin D1 protein can tie up the PCNA protein, or other proteins involved in DNA synthesis, and thus inhibit progression through S-phase. According to this hypothesis, cyclin D1 can have a positive effect on cell cycle progression by leading to inactivation of the Rb protein. At the same time cyclin D1 might inhibit progression of cells through the S-phase, by an entirely different mechanism, particularly when expressed at very high levels, by inhibiting DNA replication. The extent to which cyclin D1 exerts this inhibitory effect might depend on the relative levels of PCNA or other proteins involved in DNA synthesis in a given type of cell. However, western blot analyses of the levels of PCNA in series of cell lines indicates that the inhibitory effect of cyclin D1 in the HBL-100 cell line and the slow growth of the MDA-MB-134 cells can not be simply attributed to a deficiency of PCNA. Therefore, other potential target proteins might remain to be examined. Regardless of the underlying mechanisms, the present studies suggest that the effects of increased expression of cyclin D1 on the growth of human tumors may be highly dependent on the level of expression of other genes that play a critical role in cell cycle progression and DNA synthesis.

Materials and methods

Cell culture and viral transduction

The breast cell lines used in these studies (Hs578Bst, HBL-100, MDA-MB-134, MCF-7, MDA-MB-330, ZR-75-1 and T47-D) were purchased from the ATCC. The first two lines were originally established from apparently normal human mammary epithelium and the remaining lines from human breast carcinomas, as described in the ATCC catalog. The culture medium used for each cell line was that described in the ATCC catalog. Ten percent fetal calf serum (FCS) was added to the medium for all cell lines, unless indicated otherwise. All cell cultures were maintained in a 37°C incubator with 5% CO_2.

The construction of the cyclin D1 retroviral expression plasmid PMV7-CCND1 and the methods used for virus packaging and transduction have been previously described (Jiang *et al.*, 1993b). Briefly, viral supernatants were obtained from the PMV7 vector itself and from PMV7-CCND1 after transfection of these constructs into the amphotropic GPAM 12 packaging cell line. These viral supernatants were used to infect HBL-100 cells. Cells were selected in the presence of 1 mg ml^{-1} G418 and resistant colonies selected and expanded for further analysis.

Protein extraction and western blot analysis

Protein extractions and western blots were performed as previously described (Jiang *et al.*, 1993a) with minor modifications. Exponentially growing cells were collected with a rubber policeman and washed three times with ice-cold PBS. The cell pellets were resuspended in lysis buffer (20 mM Tris-HCl pH 7.4, 2 mM EGTA, 2 mM EDTA, 6 mMβ-mercaptoethanol 1% NP-40, 0.1% SDS and 10 mM NaF, plus the protease inhibitors aprotinin 10 μg ml^{-1}, leupeptin 10 μg ml^{-1} and PMSF 1 mM). This suspension was sonicated three times with a Sonifier Cell Disruptor. Protein concentrations were determined by the method of Lowry. For western blotting, 50 μg of protein in the total cell lysates were subjected to 10% SDS-PAGE. The proteins on these gels were transferred, using transfer buffer (25 mM Tris, 190 mM glycine, 10% methanol), to immobilin-P membranes (Millipore, Bedford, MA) at 60 volt for 3 h. Membranes were blocked with blocking buffer (50 mM Tris, 200 mM Nacl, 0.2% triton X-100, 3% BSA) for 90 min at room temperature. The membranes were then

incubated with a 1:2000 dilution of the indicated anti-human cyclin antibodies for 90 min. The polyclonal antibodies to cyclin D1, E and A were obtained from UBI (Lake Placid, NY). After washing with washing buffer (blocking buffer without 3% BSA) for 1 h, horseradish peroxidase linked anti-rabbit donkey serum (1:5000) was added for 1 h. The membranes were then washed with washing buffer for 2 h and immune detection was performed using the ECL Western blotting detection system (Amersham). The intensities of the cyclin D1 bands were quantitated by densitometric scanning.

Southern blot analysis

Genomic DNA was isolated from cells as described (Gross-Bellard *et al.*, 1973). DNA was digested with either EcoRI or HindIII for 16 h. Five μg of digested DNA were electrophoresed on a 1% agarose gel. DNA on the gel was transferred to Hybond-N membranes and hybridization to the ^{32}P-labeled cyclin D1 DNA probe was carried out as previously described (Jiang *et al.*, 1992).

Northern blot analysis

Cells from exponentially dividing cultures were collected with a rubber policeman into 50 ml tubes, washed three times with ice-cold PBS and then lysed by suspension in lysis buffer (3M LiCl and 6M urea) and homogenized for 1 min using a PCU sonicator. The tubes were placed in ice overnight at 4°C. The next day, the tubes were centrifuged and the pellets were resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) plus 0.5% SDS, followed by phenol/chloroform extraction as previously described (Favoloro *et al.*, 1980). The RNA samples (10 μg) were electrophoresed in 1% agar-6% formaldehyde gels and blotted onto Hybond-N membranes. The blotted membranes were preincubated in Church buffer at 65°C and then hybridized with ^{32}P-labelled probes to human cyclin D1, cyclin A, c-*myc* or c-*jun* for 16 h. The membranes were washed with 1 x SSC containing 0.2% SDS for 20 min at room temperature followed by washing for 20 min at 65°C, using the same washing solution. After the final wash with 1 x SSC at 65°C, the membranes were exposed to Kodak XAR-5 film with intensifying screens at −70°C.

Flow cytometric analysis

To obtain exponential cultures of HBL-100 cells or their derivatives 5×10^5 cells were plated in 10 cm dishes containing McCoy 5A medium plus 10% FCS and grown for 2 day. The cells were then trypsinized, collected and washed twice with PBS. Cell pellets were resuspended in 1 ml PBS and fixed in 5 ml of 70% ethanol and stored at 4°C. On the day of analysis, cells were collected by centrifugation and the pellets were resuspended in 0.2 mg ml^{-1} of propidium iodide containing 0.6% NP-40. RNAse (1 mg ml^{-1}) was added and the suspension was incubated in the dark at room temperature for 30 min. The cell suspension was then filtered through a 60 μM Spectra mesh filter and analysed on a Coulter EPICS 753 flow cytometer for DNA content. The percent of cells in different phases of the cell cycle was determined using a ModFit 5.2 computer program.

Growth curves and saturation densities

The exponential doubling times and saturation densities for vector control clones and the cyclin D1 overproducing clones were determined essentially as described previously (Cacace *et al.*, 1993; Jiang *et al.*, 1993b). One $\times 10^4$ cells were plated per 35 mm well in replicate 6 well plates in the presence of either 1% or 10% FCS. Every 2 days aliquots of cells were counted, up to 13 days, using a Coulter counter. Cells were refed with fresh medium every 2 days during this time course. The doubling times were calculated from the initial exponential phase of the growth curve and the saturation density from the plateau of the growth curve.

Soft-agar and tumorigenicity assays

Growth in soft-agar assays were performed as described (Han *et al.*, 1993). These assays were done in 1% FCS. For the bottom layer of agar, 1 ml of 0.5% agar was placed in each 35 mm well of 6 well plates. Then 2 ml of 0.3% top agar containing 1×10^5 cells were layered on top of the solidified layer of bottom agar. Colony formation was monitored by microscopy for up to 14 days and the final numbers of colonies that were >0.1 mm diameter then determined. Tumorigenicity assays were performed as described (Jiang *et al.*, 1993b). Briefly, cells were trypsinized, collected, and washed three times with PBS. Then 1×10^7 cells were injected subcutaneously per site (two sites per mouse) into 4 to 6 week old nude mice. Tumor development was monitored by palpation for up to 14 weeks.

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Deregulated expression of cyclin D1 and other cell cycle-related genes in carcinogen-induced rat mammary tumors

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Dysregulation of cyclin expression has been reported for several human malignancies, including breast cancer. To further investigate the role of cyclin genes in mammary tumorigenesis we analyzed the expression of cyclins D1, E and A and other cell cycle-related proteins in a series of nine *N*-methyl-*N*-nitrosourea-induced primary rat mammary tumors. Western blot analysis revealed a 10- to 15-fold increase in the level of cyclin D1 protein in most (7/9) of the tumors, when compared with normal rat mammary gland. The two tumors that did not show this increase also displayed negligible levels of the retinoblastoma protein. A moderate increase, 1.5- to 2-fold, in the level of cyclin E was observed in four tumors and three tumors displayed abnormal low molecular weight cyclin E-related proteins. None of the tumors showed amplification of the cyclin D1 or E genes when studied by Southern blot analysis. All nine tumors showed a 2- to 6-fold increase in the level of cyclin A protein. Most of the tumors also displayed a marked increase in levels of the CDK2 and CDK4 proteins. These changes did not appear to be simply a consequence of increased cell proliferation, as assessed by proliferating cell nuclear antigen analysis. Thus, aberrant expression of cyclins and other cyclin-related genes occurs frequently in mammary tumorigenesis in both rodents and humans

Introduction

There is now extensive evidence that, as originally discovered in lower organisms, the orderly progression of dividing mammalian cells through the various phases of the cell cycle is governed by a series of proteins called cyclins, which exert their effects through specific cyclin-dependent protein kinases (for reviews see 1,2). At least eight cyclin genes have been identified in mammalian cells. The G1 cyclins (C, D1–3 and E) are maximally expressed during the G1 phase of the cell cycle; cyclin A is expressed in early S phase; cyclins B1 and B2 are expressed during G2/M. Cyclins act by binding to and stimulating the activities of a series of cyclin-dependent protein kinases (CDK*). The activities of these CDKs are further modulated by protein phosphorylation and dephosphorylation and by a group of specific inhibitory proteins (3). To date, at least six mammalian CDKs (1–6) have been identified. Several studies indicate that cyclin D1 is involved in inactivating the function of retinoblastoma (Rb) protein, presumably through phosphorylation and/or the formation of a physical complex (4). Furthermore, cyclin D1 expression is positively regulated by Rb protein, thus providing an autoregulatory feedback loop (5).

There is increasing evidence that several types of human tumors display abnormalities in cyclin genes (for a review see 3). The cyclin E gene is often deregulated and overexpressed in human breast tumor cell lines and in primary human breast, colon and prostate carcinomas, but, with rare exceptions, it is not usually amplified (6–9). There are numerous examples of abnormalities in the cyclin D1 gene. This gene, also termed *prad* or *bcl-1*, is located at chromosome 11q13. Chromosomal rearrangements at this locus in parathyroid tumors or certain B cell lymphomas lead to increased expression of this gene (10). More importantly, cyclin D1 is amplified and overexpressed in a significant fraction of primary human breast carcinomas, esophageal carcinomas and several other types of tumors both in cell lines and in primary tumors (6,8,11). Our laboratory demonstrated that stable overexpression of cyclin D1 in rodent fibroblasts enhances their tumorigenicity in nude mice (12). Cyclin D1 cooperates with a defective adenovirus E1A (13) or an activated *ras* oncogene (14) in the transformation of rodent fibroblasts. Furthermore, overexpression of cyclin D1 in transgenic mice results in hyperplasia and tumors of the mammary epithelium (15). Thus overexpression of cyclin D1 appears to play a critical role in mammary carcinogenesis.

In view of the above findings in human tumors it was of interest to determine whether carcinogen-induced rat mammary carcinomas also display abnormalities in the expression of cyclins and cyclin-related genes. The present study demonstrates that primary rat mammary carcinomas induced by *N*-methyl-*N*-nitrosourea (NMU) frequently show increased expression of cyclins D1, E and A and CDKs 2 and 4 and that these changes do not appear to be due simply to increased cell proliferation. Thus this model system appears to resemble human mammary carcinogenesis and may be useful for mechanistic studies on deregulated expression of cyclin genes.

Materials and methods

Animals

The source and housing of the animals used in this study have been described previously (16). Mammary cancers were induced in 50-day-old, virgin female Sprague–Dawley rats with a single i.v. injection of 50 mg NMU/kg body wt via the jugular vein, as described (16). A total of 12 rats was used in this study.

Ten rats were treated in this way. The other two received only a single i.v. injection of the acidified saline solution used to dissolve the NMU, as controls. The rats were followed for a total of 120 days, a time of high incidence of malignant tumors after NMU administration (17). At that time seven of the 10 treated rats showed tumors and two of these seven showed two tumors each. No tumors were observed in control animals.

A total of nine tumors were analyzed. They were all malignant tumors with a predominant cystic papillary adenocarcinoma pattern. The tumors were classified as reported (18). A summary of the major features of the nine tumors is presented in Table I.

***Abbreviations:** CDK, cyclin-dependent protein kinase; NMU, *N*-methyl-*N*-nitrosourea; Rb, retinoblastoma; PCNA, proliferating cell nuclear antigen.

Table I. Histopathological features of NMU-induced rat mammary tumors

Tumor number[a]	Histological type	Tissue invaded	Tumor size[b]	Other features
1	Papillary	Fat	++	Small amount of necrosis
2	Papillary	Connective	+++	Highly differentiated
3	Papillary	Connective	+	Cribriform component
4	Papillary	Muscle	++	Highly differentiated
5	Cribriform	NE[c]	++	Solid component
6	Papillary	Muscle	+++	
7	Papillary	NE	+	Cribriform component
8	Papillary	Fat	++	
9	Papillary	Muscle	++	

[a]Tumors 1 and 2 were from the same rat, as were tumors 8 and 9. Each of the remaining tumors were from separate rats.
[b]Tumor size was based on the approximate tumor diameter: +++ = 15 mm; ++ = 10 mm; + = 5 mm.
[c]NE designates not evaluated, because normal tissue was not present in these samples.

The normal mammary glands were collected from the two untreated non-pregnant rats that were of the same strain and age as those of the tumor-bearing rats used in this study. Multiple glands were collected from each of the two untreated rats, immediately frozen and used as a normal control. Protein extracts from a pool of glands from each rat were examined and gave similar results.

DNA analysis

High molecular weight DNA was isolated from tumors by standard proteinase K digestion and phenol-chloroform extraction, as described previously (11). After quantification by UV spectrometry, DNA (10 μg) was digested overnight with the restriction enzymes *Bam*HI or *Hin*dIII (Boehringer Mannheim), electrophoresed in a 0.8% agarose gel, transferred onto a nylon membrane (Hybond-N; Amersham) by the Southern blot method and cross-linked by UV light. Filters were hybridized to DNA probes (see below), [^{32}P]dCTP-labeled using a Multiprime DNA labeling system (Amersham). Hybridization was performed at 65°C overnight in Church buffer (19). After washing, the blots were exposed to Kodak XAR-5 film at −70°C in a cassette with intensifying screens.

Probes

The cyclin D1 cDNA probe was prepared as previously described (11). The cyclin E cDNA probe was obtained as a *Hin*dIII−*Hin*dIII fragment from the vector LXSN-cyclin E (20). Both of these cDNA probes are human, but they cross react with both mouse and rat species (12,20). Ethidium bromide staining was used as a control for equal loading of samples in the gel. For cyclin D1 an H-*ras* probe was also used as an internal control (11). The pattern obtained with the H-*ras* probe correlated with the results of the ethidium bromide staining (data not shown).

Western blot analysis

Frozen tissue from each tumor and from normal mammary glands was added to 3–5 volumes of sonication buffer containing proteases and phosphatase inhibitors (20 mM Tris−HCl pH 7.4, 2 mM EGTA, 6 mM β-mercaptoethanol, 1% NP-40, 0.1% SDS, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) and sonicated at 4°C with a Sonifier Cell Disruptor. Homogenates were incubated on ice for 30 min and then centrifuged. The supernatants were assayed for protein content by the Lowry method and stored at −70°C.

For Western blotting, 50 μg of protein from each tissue sample were electrophoresed in each lane of a 10% SDS−polyacrylamide gel and then transferred to immobilon-P membranes (Millipore) at 60 V for 3 h at 4°C in transfer buffer (25 mM Tris, 190 mM glycine, 10% methanol). Blots were then incubated with blocking buffer (50 mM Tris, 200 mM NaCl, 0.2% Triton X-100, 3% bovine serum albumin) for 90 min at room temperature. Different dilutions were used for different primary antibodies. Anti-cyclin D1 antibody was used as already reported (16). To detect cyclin E, two different antibodies were used and gave similar results: a rabbit polyclonal anti-cyclin E from UBI and a mouse monoclonal anti-cyclin E from Pharmingen. Rabbit polyclonal antibodies to cyclin A (UBI), CDK2 (UBI) and CDK4 (Santa Cruz) and a monoclonal antibody to Rb protein (Pharmingen) were used as suggested by the suppliers. Immunodetection was performed using the enhanced chemiluminescence kit for Western blotting detection (Amersham). Film exposure ranged from a few seconds to 20 min, depending on the primary antibody. Bands were quantitated on a Molecular Dynamics computing densitometer using ImageQuant software, version 3.22. Protein extraction was independently performed twice for each tumor sample. For the normal tissue, protein extracts were prepared from a pool of normal mammary glands from each of the two control animals. Similar results were obtained when the two protein extracts from each sample were tested with the same antibodies. For cyclin E, the densitometric data shown in Figure 1B are the mean value of the results obtained with the polyclonal and the monoclonal antibodies to cyclin E.

Results

Expression of cyclins D1, E and A in rat mammary tumors

In the present study we examined a series of nine primary rat mammary tumors induced by NMU for the expression of cyclin D1 and related genes that have been reported to be deregulated in human breast tumors (see Introduction). Western blot analysis revealed that cyclin D1 was present at a very low level in normal rat mammary gland, but was increased 3- to 15-fold in all of the nine rat mammary tumors examined in this study (Figure 1). We performed Southern blot analysis to determine if cyclin D1 deregulation was related to gene amplification, which is often seen with human breast tumors (6,10), but none of the rat mammary tumors showed significant evidence of cyclin D1 gene amplification, compared with normal rat genomic DNA, when the data were normalized for equal amounts of DNA (Figure 2).

To determine whether the increased expression of cyclin D1 was simply due to increased cell proliferation, we assessed the proliferative activity of the tumors by measuring the expression of proliferating cell nuclear antigen (PCNA) by Western blot analysis. Although all of the tumor samples also displayed increased levels of PCNA, there was no simple correlation between the levels of PCNA and cyclin D1 (Figure 1). For example, tumor 3 had the highest level of cyclin D1, but only a moderate increase in PCNA (Figure 1). Similar findings have been reported for human breast cancer (21,22).

Dysregulation of the expression of another G1 cyclin, cyclin E, has also been seen in certain human tumors, including breast cancer (6,8,9). Amplification of the cyclin E gene has been reported as a rare occurrence in human breast and colon carcinomas (6–8). In human breast cancer the increased level of expression of cyclin E has been related with tumor grade and stage (9). Western blot analysis showed a moderate increase in the level of cyclin E in the rat mammary tumors 2, 3, 5 and 6, when compared with normal mammary gland (Figure 1). Again, there was no correlation with the level of PCNA, or the level of cyclin D1. The cyclin E antibody recognized one major band with the expected size of ~55 kDa in the normal mammary gland sample. In tumors 6, 8 and 9 the cyclin E antibody also recognized a band migrating at ~42 kDa (Figure 1). This band was recognized by both polyclonal and monoclonal anti-cyclin E antibodies (data not shown). In addition, these two antibodies recognized another band, at

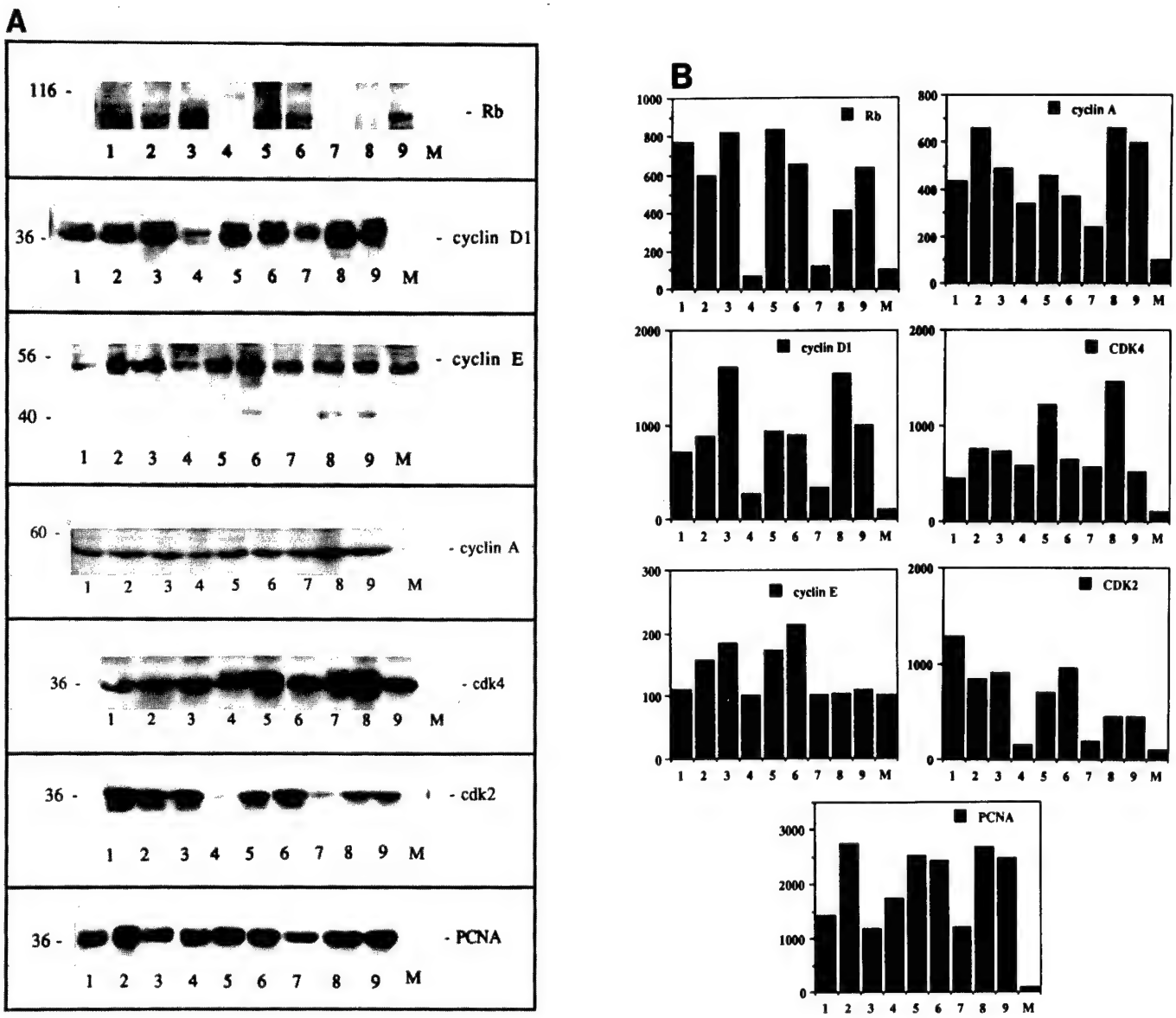

Fig. 1. Deregulated expression of cyclins and cyclin-related proteins in rat mammary tumors. **(A)** Protein extracts from NMU-induced rat mammary tumors (1–9) or from normal rat mammary gland (M) were used to perform Western blot analysis as described in Materials and methods, using the antibodies to the indicated proteins. The position of molecular weight markers in kilodaltons (kDa) are indicated on the left. Faint bands for cyclin D1, PCNA, Rb protein and the two CDKs were seen on the original Western blots of the normal mammary gland sample. **(B)** Intensity of the corresponding bands determined by densitometer analysis.

~36 kDa, which was present in all of the tumors and was not present in normal tissue (data not shown). Lower molecular weight cyclin E related proteins have also been seen in human breast cancers and other types of human cancer (9). Southern blot analysis revealed no evidence of significant amplification of the cyclin E gene in any of the tumor samples when compared with normal rat genomic DNA (Figure 2).

Increased expression of cyclin A in the absence of amplification has also been reported in human breast cancer, in both primary tumors (8) and in cell lines (6,8). All of the rat mammary tumors displayed an ~2- to 6-fold increase in the 58 kDa cyclin A protein when compared with the normal mammary gland. No correlation was observed between cyclin A expression and the proliferative activity of the tumors as assessed by the level of PCNA expression (Figure 1).

Expression of cyclin-dependent kinases in rat mammary tumors

Cyclins act as key regulators of cell cycle progression by binding to and activating a series of cyclin-dependent protein kinases. Thus, as recently suggested (3), altered expression of CDKs may also be involved in tumorigenesis. The two major CDKs that interact with cyclins D1, E and A are CDK2 and CDK4 (1,2), so it was also of interest to evaluate their expression in these tumor samples.

Western blot analysis showed increased expression of both CDK4 and CDK2 in most of the tumors. A marked increase in expression of the 34 kDa CDK4 protein was observed in all of the tumors when compared with the normal mammary gland (Figure 1). Seven of the nine tumors also displayed increased levels of the 33 kDa CDK2 protein (Figure 1). Amongst the tumors there was no simple correlation between

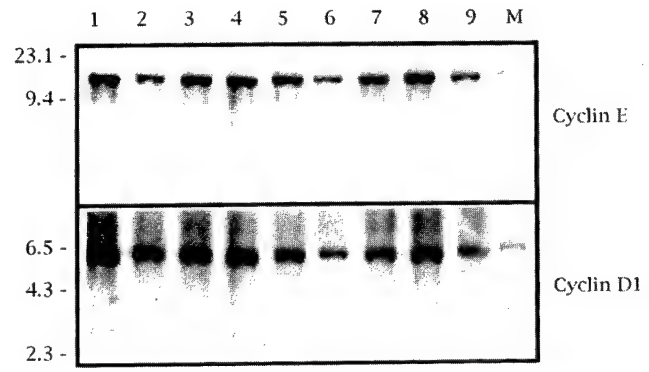

Fig. 2. Southern blot analysis of cyclin E (top) and cyclin D1 (bottom) on DNA samples from rat mammary tumors. Genomic DNA (10 μg) samples from each tumour (1–9) or from normal rat mammary tissue (M) were digested with *Bam*HI, separated by electrophoresis on an agarose gel, transferred to a nylon filter and hybridized to [^{32}P]-labeled cyclin E or cyclin D1 probes, as described in Materials and methods. The positions of molecular weight markers (in kilobases) are indicated on the left. Ethidium bromide staining of the gel indicated that less DNA was present in the M sample.

the levels of CDK4 and CDK2. Thus tumor 8 had the highest level of CDK4, but did not have the highest level of CDK2 (Figure 1).

Tumor suppressor genes in rat mammary tumors

Because of the known complex interaction between cyclin D1 and the Rb tumor suppressor gene (see Introduction and Discussion), we also examined the level of expression of the Rb protein in these samples. Western blot analysis indicated a very low level of the 105 kDa Rb protein in the normal mammary gland sample and a marked increase in the level of this protein in seven of the nine tumor samples (Figure 1). It is of interest that the two tumors (nos 4 and 7) that displayed low or undetectable levels of the Rb protein also displayed very low levels of the cyclin D1 protein (Figure 1). These findings are consistent with those seen in human tumors (21,23) and suggest that in rat mammary cells, as in human mammary cells (21), the Rb−cyclin D1 pathway is a frequent target of oncogenic alterations (see also Discussion).

Discussion

The present study indicates that increased expression of cyclins D1, E and A and of CDK2 and CDK4 occurs frequently in NMU-induced primary rat mammary tumors when compared with age-matched normal mammary gland tissue. The most striking changes were seen with cyclin D1. We should emphasize that the interpretation of these findings is complicated by two factors. The first is the fact that the tumor tissue represents largely replicating cells, whereas the normal tissue represents largely non-replicating cells. This is apparent from the results obtained on the immunoblot for PCNA (Figure 1), which have been confirmed by immunostaining for PCNA (data not shown). Nevertheless, amongst the tumors there was no simple correlation between the abundance of cyclin D1 and PCNA, nor was there a correlation between the abundance of cyclin D1 and cyclin E or cyclin A (Figure 1). In additional studies (not shown here) we also found, by immunostaining, a lack of correlation between cyclin D1 and PCNA staining amongst the tumor sample. A second factor is that in the normal mammary gland only a small fraction of the cells are mammary epithelial cells and the remainder are adipose and stromal cells, whereas the tumor samples contain mainly epithelial carcinoma cells. However, in immunohistochemical staining studies we have also found that there is negligible expression of cyclin D1 in normal mammary epithelial cells and in the other cells present in the normal mammary gland samples. On the other hand, the mammary carcinoma cells in the tumor samples analyzed in the present study displayed moderate to strong nuclear immunostaining for cyclin D1, whereas the relatively low percentage of stromal cells showed negligible staining. Thus tumor samples that displayed high levels of cyclin D1 by Western blot analysis (for example tumor 3; Figure 1) displayed strong nuclear staining for cyclin D1 in the carcinoma cells and negligible staining in adjacent stromal cells (data not shown). Therefore, the results obtained in the present study do not appear to be due simply to differences between normal mammary gland and tumors in the relative amounts of epithelial and stromal cells.

The heterogeneity of the profiles of expression of cyclin D1, E and A and of CDK2 and CDK4 between the individual tumors (Figure 1) is consistent with previous evidence that, although induced by the same carcinogen in the same species, a series of primary tumors can display considerable inter-tumor heterogeneity for various properties (26). Although our sample size is small, there was no obvious correlation between the histopathology of individual tumors and the levels of expression of the above-described proteins (Table I and Figure 1).

Southern blot analysis (Figure 2) of the genomic DNA of these tumors indicated that the increased expression of cyclin D1 and E was not due to amplification of the corresponding genes. Studies on primary human breast cancers indicate that ~15–20% display amplification and increased expression of cyclin D1, but an additional ~20% of these tumors display increased expression of cyclin D1, which cannot be attributed to gene amplification (6,22). The increased expression of cyclin E and cyclin A often seen in human breast and other tumors is also not usually associated with gene amplification (6–8). It is of interest that both rat mammary tumors (Figure 1) and human breast tumors (8,9) often display lower molecular weight cyclin E proteins, but the significance of this finding is not known.

Further studies are required to determine whether the increased expression of certain cell cycle-related proteins in both human and rat mammary tumors reflects changes at the level of *de novo* gene transcription, mRNA stability, protein translation or protein turnover. In tumors in which this is not due to gene amplification or mutations these changes may be secondary to mutations in oncogenes or tumor suppressor genes. In contrast to human breast tumors, which often display mutations in the *p53* gene (25), carcinogen-induced rat mammary tumors do not usually show *p53* mutations (26). Most of the rat tumors also expressed the Rb protein (Figure 1). Therefore, it is unlikely that mutations in the *p53* or Rb genes play a role in the altered expression of the above-mentioned genes in rat mammary tumors.

Activating mutations in the c-H-*ras* gene occur in ~75% of NMU-induced rat mammary tumors (27,28). A recent study indicates that transformation of the rat enterocyte cell line IEC-18 with an activated human H-*ras* gene induces increased expression of cyclin D1 (29) and our laboratory has obtained similar results with a c-K-*ras* oncogene (unpublished studies). Thus it is possible that some or all of the changes described in the present study are secondary to NMU-induced mutations

in the c-H-*ras* oncogene. However, other mechanisms must be operative in human breast tumors, since only rarely do they display mutations in *ras* genes (30).

To our knowledge, the only previous studies on cyclin gene expression in carcinogen-induced rodent tumors are two studies on mouse skin carcinogenesis, each of which employed different protocols. In a two-stage model in which tumors were initiated with a single application of 7,12-dimethylbenz[*a*]-anthracene and then promoted with repetitive applications of 12-*O*-tetradecanoylphorbol-13-acetate, cyclin D1 amplification and overexpression was detected both in carcinomas and dysplastic papillomas (31). On the other hand, when mouse skin tumors were induced by repetitive applications of benzo[*a*]pyrene, no amplification nor overexpression of cyclin D1 gene was observed (32). It is of interest that in the former study increased expression of cyclin D1 is a fairly early event, since it was seen in the advanced papillomas (31). It remains to be determined how early this change occurs in mammary carcinogenesis. The first protocol used to induce skin tumors is associated with activating mutations in the c-H-*ras* oncogene (33), but this is not the case with the second protocol (32). These findings are consistent, therefore, with the above-mentioned hypothesis that the increased expression of cyclin D1 in the NMU-induced rat mammary tumors seen in the present study is a consequence of *ras* gene activation.

There is increasing evidence that increased expression of cyclin D1 contributes to the process of cell transformation and tumor development. Thus overexpression of cyclin D1 in rodent fibroblasts can enhance cell transformation either alone (12) or in combination with an activated *ras* oncogene (14) or a defective adenovirus E1A gene (13). Furthermore, overexpression of cyclin D1 in the mammary gland of transgenic mice leads to mammary hyperplasia and mammary tumor formation (15), a finding directly relevant to the present study.

In the present study we also observed increased levels of the cyclin E protein and/or lower molecular weight forms of this protein in about half of the NMU-induced rat mammary tumors (Figure 1). Similar changes have been previously reported in some human breast cancer cell lines and in primary human breast cancers (6,8,9). In the latter studies these changes were associated with increasing grade and stage of the tumors (9). Since these changes in the rat mammary tumors were also not associated with amplification of the cyclin E gene, like the increased expression of cyclin D1, they are presumably a consequence of other events. Nor was there an apparent correlation amongst the individual tumors between changes in cyclin E, cell proliferation or increased expression of cyclin D1. Thus the basis for the changes in cyclin E expression remains to be determined.

Taken together, the present study indicates that the changes in the expression of cyclins D1, E and A, originally described in human breast cancer, extend to primary mammary tumors induced in rats by the chemical carcinogen NMU. As mentioned above, it has also been shown that overexpression of cyclin D1 in the mammary gland of transgenic mice causes mammary hyperplasia and mammary tumors (15). Thus it would appear that altered expression of cyclin D1 and other cyclins is fundamental to the process of mammary carcinogenesis in both rodents and humans.

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Increased Expression of Cyclin D1 in a Murine Mammary Epithelial Cell Line Induces p27^{kip1}, Inhibits Growth, and Enhances Apoptosis[1]

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Abstract

Cyclin D1 is frequently amplified and/or overexpressed in human breast cancer and several other types of cancer. To examine the role of cyclin D1 in normal mammary epithelial cells, in the present study we have overexpressed human cyclin D1 in the mouse mammary epithelial cell line HC11, using retrovirus-mediated transduction. We found that the cyclin D1 overexpresser clones displayed a decrease in saturation density, a decrease in anchorage-independent growth, an increased fraction of cells in the G_0-G_1 phase, and increased expression of β-casein, when compared to the control cells. The latter finding suggested that they were more differentiated. Furthermore, the cyclin D1 overexpressers displayed a marked increase in susceptibility to induction of apoptosis by serum withdrawal or by treatment with hydroxyurea or the protein kinase C inhibitors CGP 41251 and Ro31-8220. Thus, in some mammary epithelial cells, increased expression of cyclin D1 can inhibit growth, induce differentiation, and enhance apoptosis. These effects might be due, at least in part, to the fact that these derivatives displayed increased expression of the p27^{kip1} inhibitory protein.

Introduction

Cell cycle progression in eukaryotic cells is governed by a series of cyclins and cdks.[3] Individual cyclins act at different phases of the cell cycle by binding to and stimulating the activities of a series of cdks. Because these cyclins and cdks are pivotal to cell cycle control and thereby cell proliferation, mutational changes and alterations in expression of the corresponding genes can play a critical role in tumorigenesis (reviewed in Refs. 1–3).

A number of cyclin genes (*A, B, C, D1–3, E,* and *H*) and cdk genes (*cdk1–cdk7*) have been identified in mammalian cells (1, 3). The G_1 cyclins (*C, D1–D3,* and *E*) complex with their cdk partners and regulate progression of the G_1 phase. Cyclin D1 can complex with cdk4, cdk5, or cdk6 to regulate the early to mid-G_1 phase of the cell cycle (4–6). The cyclin E-cdk2 and cyclin A-cdk2 complexes control the G_1-S transition and the S phase, respectively (7–12). Cyclins B1 and B2 complex with cdk1 (also termed cdc2) and control the G_2-M phase (13). Recently, a number of cdkIs have been identified, and their functions have been analyzed. The *MTS/INK* gene family members *p15, p16, p18,* and *p19* inhibit the activities of the cyclin D-cdk4 and cyclin D-cdk6 complexes (14–18). The *p21* gene, also known as *WAF1/CIP1/Sdi1/CAP20* (hereafter referred to as *p21*^{WAF1}), regulates the activities of the cyclin D-cdk4, cyclin A-cdk2, and cyclin E-cdk2 complexes (19–23). The *Kip* gene family members *p27* and *p57* control the activities of the cyclin D-cdk4, cyclin A-cdk2, and cyclin E-cdk2 complexes (24–28). Several types of human tumors display abnormalities in cyclin and cdk genes. The *cyclin A* gene was the site of integration of the hepatitis B virus in a human hepatocellular carcinoma (29). The *cyclin E* gene is often deregulated and overexpressed in several human tumors, including breast, colon, and prostate carcinomas (30–32). The *cyclin D1* gene is amplified and overexpressed in primary human breast carcinomas (33–34), esophageal carcinomas (35–36), and several other types of tumors (37–40). Furthermore, when cyclin D1 was overexpressed in transgenic mice, mammary hyperplasia and tumors of the mammary epithelium occurred (41). The *cdk4* gene is frequently amplified in human glioblastomas (42, 43). The *INK/MTS p16* gene is deleted, mutated, or hypermethylated in a number of human tumors (15, 44–47). Mutations in the *p53* tumor suppressor gene impair the induction of *p21*^{WAF1} in response to DNA damage, and mutations in the *p21*^{WAF1} gene occur in Burkitt's lymphoma (48).

To gain further insight into the role of the *cyclin D1* gene in growth control, investigators have overexpressed the corresponding cDNA in fibroblasts (49, 50). This resulted in a shorter G_1 phase and, in some cases, enhancement of growth and cell transformation. In contrast, we recently reported that stable overexpression of cyclin D1 in the human mammary epithelial cell line HBL-100 inhibited growth and lengthened the S phase (51). These findings suggest that cyclin D1 can have different effects on growth control, depending on the cell type.

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[3] The abbreviations used are: cdk, cyclin-dependent kinase; cdkI, cdk inhibitor; GST-Rb, glutathione *S*-transferase-retinoblastoma protein; DAPI, 4′,6-diaminidino-2-phenylindole; PKC, protein kinase C.

To further explore the role of cyclin D1 in mammary tumorigenesis, in the present study cyclin D1 was stably overexpressed in the normal mouse mammary epithelial cell line HC11, using retrovirus-mediated gene transfer. The HC11 cell line was originally established from a normal mouse mammary gland. Although it is an immortalized cell line, it has an epithelial morphology and normal growth properties and is not tumorigenic (52, 53). Furthermore, it secretes the milk protein β-casein upon stimulation with growth hormones (53–55). We found that cyclin D1 overexpression in these cells slightly inhibited their growth and resulted in a lengthening, rather than shortening, of the G_1 phase. It also increased the expression of cyclins E and A, the $p27^{kip1}$ protein, and casein, a marker of mammary epithelial cell differentiation. Overexpression of cyclin D1 in the HC11 cells and in the human HBL-100 mammary epithelial cells also enhanced apoptosis, especially after serum starvation or treatment with various drugs. These findings suggest a role for cyclin D1 in differentiation and in programmed cell death in mammary epithelial cells.

Results

Development of Derivatives of the HC11 Cell Line That Stably Express High Levels of Cyclin D1. A human *cyclin D1* cDNA was stably overexpressed in the normal mouse mammary epithelial cell line HC11 by using a retroviral expression vector (pMV7-CCND1) that was previously described and studied in rat R6 fibroblasts (49). After infection and selection for neomycin resistance (see "Materials and Methods"), a number of vector control and cyclin D1 clones were obtained. Protein extracts from exponentially growing cultures were obtained and analyzed on SDS-PAGE as described in "Materials and Methods." All of the cyclin D1 clones expressed increased amounts of the cyclin D1 protein, at varying levels, as determined by Western blot analysis (data not shown). Cyclin D1 protein expression in four of the cyclin D1 clones and in two of the vector control clones is shown in Fig. 1. A relatively low level of the cyclin D1 protein was detected in the parental HC11 cells and the two vector controls (HC11-vt#3 and HC11-vt#4) by Western blot analysis. Densitometric analysis of Western blots indicated that the HC11 cyclin D1 clones (HC11-D1#5, D1#7, D1#19, and D1#20) expressed about an 8-fold increase in cyclin D1 protein when compared to the two vector control clones. A lighter exposure of the film indicates that the exogenous human cyclin D1 protein is a single band (data not shown). Northern blot analysis demonstrated a high level of the exogenous *cyclin D1* mRNA, of the expected size (4.5 kb), in these four clones but not in the parental and the two vector control clones (Fig. 2). The parental and the vector control cells expressed a low level of an endogenous cyclin D1 mRNA that was slightly lower than the exogenous cyclin D1 mRNA (Fig. 2).

Effect of Cyclin D1 Overexpression on the Expression of Other Cell Cycle-related Genes. To determine whether cyclin D1 overexpression alters the expression of other cyclins, protein extracts from exponentially growing cells were isolated and examined by Western blot analysis, using cyclin E and A antibodies (Fig. 1). A cyclin E protein of about M_r 52,000 was expressed at higher levels in the cyclin D1 overexpressers than in the parental and the two vector control cell lines. Two additional lower-molecular-weight bands at about M_r 42,000–50,000 were also observed in the cyclin D1 overexpresser cells. There have been reports suggesting that similar lower-molecular-weight cyclin E proteins frequently seen in tumor cells might be due to protein degradation or alternate splicing of *cyclin E* mRNA (12, 31, 32, 57). The M_r 58,000 cyclin A protein was also expressed at higher levels in the cyclin D1 overexpressers (Fig. 1). There was an additional cyclin A band that ran just below the previous cyclin A protein band. Although the exact nature of this additional band is not known, it is possible that it represents a less phosphorylated cyclin A protein.

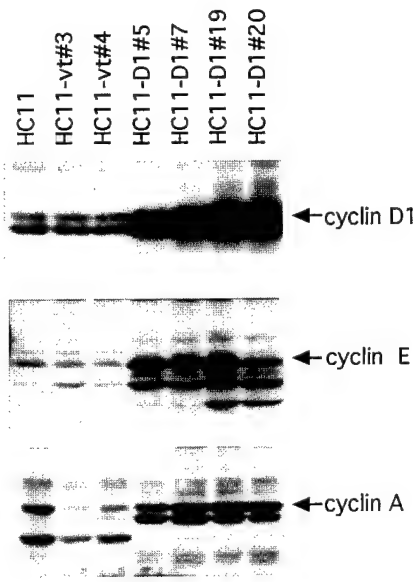

Fig. 1. Western blot analysis of protein extracts (50 μg) from the control HC11 cells (HC11-vt#3 and HC11-vt#4) and the derivatives that overexpress cyclin D1 (HC11-D5, HC11-D7, HC11-D19, and HC11–20), using antibodies to cyclin D1, cyclin E, and cyclin A, as indicated. For additional details, see "Materials and Methods."

To determine whether cyclin D1 overexpression alters the expression of cyclins D2 and D3, we performed Northern blot analysis using cyclin D2- and cyclin D3-specific probes. As shown in Fig. 2, the levels of *cyclin D2* and *cyclin D3* mRNAs were not appreciably affected by cyclin D1 overexpression. Furthermore, the levels of expression of cyclin D2 and cyclin D3 proteins were not altered when we used a pan-specific antibody that recognizes all three forms of cyclin D1 (data not shown).

Because cyclin D1 overexpression in HC11 cells induced expression of the cyclin E and cyclin A proteins, we examined the expression of the catalytic partner of these cyclins, namely cdk2. As shown in Fig. 3, the level of expression of this protein was also increased in the cyclin D1 overexpressers. However, the level of expression of the cdk4 protein, which complexes with and is activated by cyclin D1, was not altered in cyclin D1 overexpresser clones (Fig. 3). We also examined the levels of expression of the CDKI $p27^{kip1}$ and were surprised to find that the level of this protein was

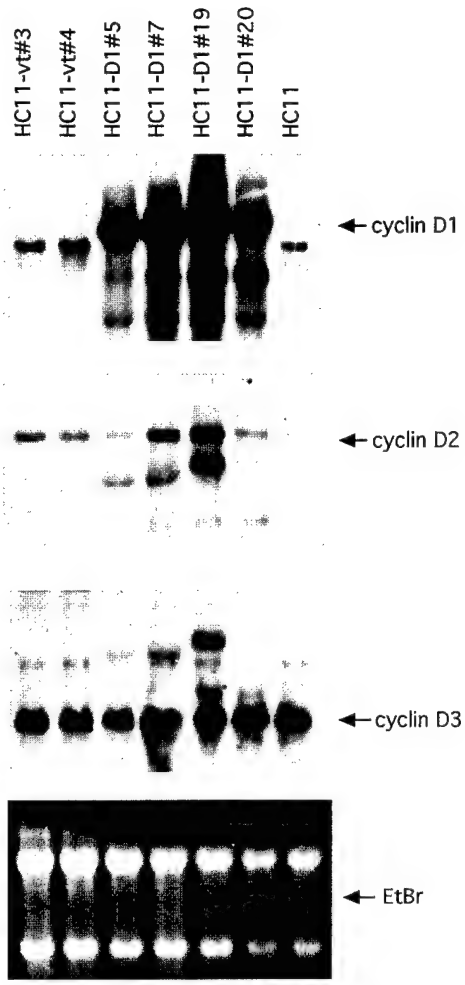

Fig. 2. Northern blot analysis of cyclin D1, cyclin D2, and cyclin D3 mRNAs. Total RNA was isolated from the control HC11 cells and the cyclin D1 overexpresser derivatives. Samples (10 μg each) were subjected to agarose gel, transferred to a membrane, and hybridized with ^{32}P-labeled human cyclin D1, mouse cyclin D2, or mouse cyclin D3 probes. Two vector control clones (HC11-vt#3 and HCT11-vt#4) and three cyclin D1 overexpresser clones (HC11-D1#5, #7, #19, and #20) are shown. Representative ethidium bromide staining of the membrane is shown as a control for RNA loading. For additional details, see "Materials and Methods."

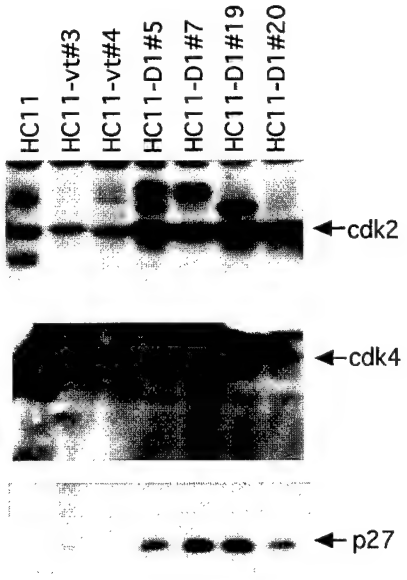

Fig. 3. Western blot analysis of protein extracts (50 μg) from the control HC11 cells and cyclin D1 derivatives using antibodies to cdk2, cdk4, and p27^{kip1}. For additional details, see "Materials and Methods."

markedly increased in the cyclin D1 clones (Fig. 3). For reasons that were not apparent, we could not detect the p16^{INK4} and p21^{WAF1} proteins in these cells by Western blot analysis. Northern blot analysis revealed that in the cyclin D1 overexpresser clones, the level of expression of the CDKI p16^{INK4} was slightly increased and that there was no change in the level of p21^{WAF1} when compared to the control cells (data not shown).

Cyclin D1 and cdk2-associated Kinase Activities in Cyclin D1-overexpressing Cells. It is known that p27^{kip1} inhibits cyclin D-cdk4, cyclin A-cdk2, and cyclin E-cdk2 kinase activities (24–26). Because the cyclin D1 overexpresser clones had a higher level of the cdkI p27^{kip1} protein, we examined cyclin D1- and cdk2-associated kinase activities. As shown in Fig. 4*A*, *in vitro* assays of GST-Rb phosphorylation by the cyclin D1 immunoprecipitates was increased in the cyclin D1 overexpresser HC11-D1#19 cell, when compared to the vector control cells. Similar results were obtained when anti-cdk4 immunoprecipitates were used in *in vitro* GST-Rb protein phosphorylation assays (data not shown). This demonstrates that the exogenous cyclin D1 is complexed to a CDK (probably cdk4) and is, therefore, probably functional *in vivo* in the derivatives of HC11 cells, despite the increase in p27^{kip1}. Thus, the growth inhibition resulting from cyclin D1 overexpression in HC11 cells is probably not due to inhibition of cyclin D1-associated kinase activity.

When cdk2 was immunoprecipitated and the associated kinase activity assayed *in vitro*, using histone H1 as a substrate, there was no significant difference between the vector control and the cyclin D1 overexpresser cells (Fig. 4*B*), despite the increased levels of cyclins E and A and cdk2 in the overexpresser cells (Fig. 3). This finding reflects the increased level of the p27^{kip1} inhibitor in the overexpresser cells.

We also examined the levels of expression and extent of *in vivo* phosphorylation of the Rb protein in these cells, by Western blot analysis, using a anti-Rb antibody. As shown in Fig. 4*C*, both the vector control and cyclin D1 overexpresser cells expressed the Rb protein, and three of the four overexpresser clones expressed an increase in the more slowly migrating hyperphosphorylated form of Rb. These results are consistent with the above-described finding of increased *in vitro* cyclin D1-associated phosphorylation of a GST-Rb substrate (Fig. 4*A*).

Alterations in Cell Growth in the Cyclin D1-overexpressing HC11 Clones. In view of the above-described changes in the expression of various cell cycle-related

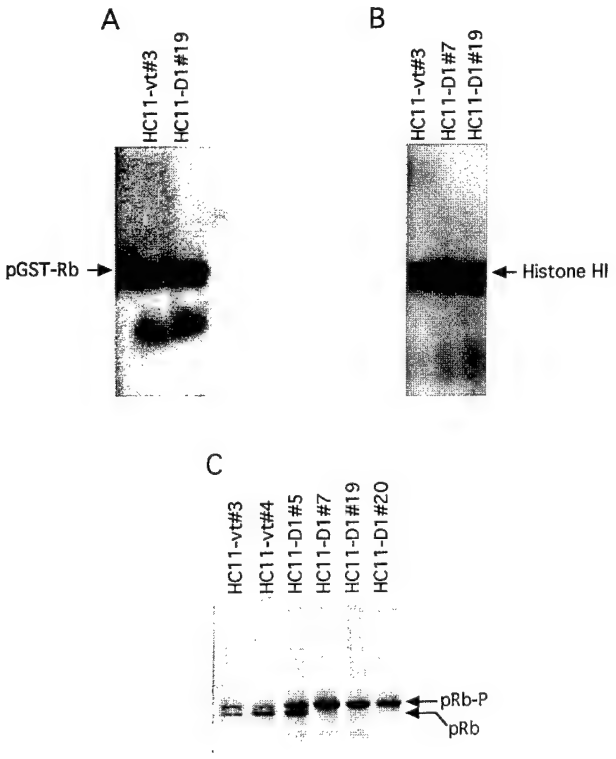

Fig. 4. In vitro assays for cyclin D1- and cdk2-associated kinase activities and the Western blot analysis for the Rb protein expression in the vector control and cyclin D1 overexpresser clones. *A* and *B*, autoradiograms of cyclin D1-associated kinase activity, in which GST-Rb is used as a substrate, and of cdk2 kinase activity, in which histone H1 is used as a substrate, respectively. *C*, Western blot of the Rb protein in the vector control and cyclin D1 overexpresser clones. For additional details, see "Materials and Methods."

genes, we examined several growth properties of the cyclin D1 overexpressers, including exponential doubling time, saturation density, and anchorage-independent growth in agar (Table 1). When grown in monolayer culture with 10% FCS, the parental HC11 cells and the two vector control clones showed similar exponential doubling times (about 14–15 h). The doubling times of the four cyclin D1 overexpressers were similar to those of the vector controls. The exponential doubling times of both the vector control and the four cyclin D1 overexpressser clones were increased (to about 36 h) when these clones were grown in the presence of 1% FCS (Table 1). The doubling times of the control and overexpresser clones were also similar when they were grown in 10% FCS in the absence of epidermal growth factor and insulin (data not shown). The saturation densities of the four cyclin D1 overexpresser clones were slightly reduced, by about 25%, when compared to the vector control clones. The parental and the vector control cells and the cyclin D1 overexpresser clones were able to grow in soft agar, but with a very low cloning efficiency. The cyclin D1 overexpressers showed a lower (about 38% decrease) in cloning efficiency in soft agar.

The above studies indicate that an approximately 8-fold increase in the expression of the cyclin D1 protein in HC11 cells did not alter their doubling time, but it did cause some

Table 1 Growth properties of cyclin D1-overexpressing HC11 cells

Statistical analyses show that *P*s for doubling times are $P > 0.10$ (1% FCS) and $P < 0.05$% (10% FCS), respectively. *P*s for saturation density and growth in agar are $P < 0.10$ and $P < 0.01$, respectively. For additional details see "Materials and Methods."

Cell line	Growth in monolayer culture			Growth in agar
	Doubling time (h)		Saturation density ($\times 10^6$)	
	1 % FCS	10% FCS	10% FCS	
HC11 - vt#3	37.0	14.1	8.5	0.8
HC11 - vt#4	36.1	14.1	8.4	0.8
Mean	36.6	14.1	8.5	0.8
HC11 - D1#5	35.2	16.3	7.7	0.5
HC11 - D1#7	41.3	15.5	7.5	0.5
HC11 - D1#19	34.0	14.9	5.4	0.5
HC11 - D1#20	34.8	14.9	5.1	0.4
Mean	36.3	15.4	6.4	0.5

decrease in saturation density and in cloning efficiency in soft agar.

Effect of Cyclin D1 Overexpression on Cell Cycle Parameters. Because cyclin D1 plays a critical role in cell cycle progression, especially in the mid- to late G_1 phase (49, 50, 58), cell cycle parameters were examined by flow cytometry in the HC11 control and cyclin D1 overexpresser clones. Cells were prepared from exponentially dividing nonsynchronized cultures, and DNA was labeled with propidium iodide and analyzed by flow cytometry.

As shown in Table 2, it is apparent that overexpression of cyclin D1 increased the percentage of the total cell population that was in the G_0-G_1 phase, decreased the percentage in S phase, and had no consistent effect on the percentage in the G_2-M phase. Although there were some variations between individual vector control and overexpresser clones (Table 2), this general pattern was also seen in two additional experiments (data not shown). Because the cyclin D1 overexpresser and control cells have similar doubling times, we also used the flow cytometry data and the exponential doubling times to calculate the approximate length in hours of each of these three phases of the cell cycle in the various cell lines (Table 2). It can be seen that the length of the G_0-G_1 phase for the cyclin D1 overexpressers was longer than that for the control clones, about 8.0 h *versus* 3.6 h, respectively. However, the length of the S phase for the four cyclin D1 overexpressers was shorter than that for the control clones, about 5.8 h *versus* 8.7 h, respectively. The length of the G_2-M phase was similar in the control cells and the overexpressers, 1.9 h *versus* 1.6 h, respectively. Thus, overexpression of cyclin D1 in HC11 cells resulted in a longer G_1 phase and concomitant shortening of the S phase.

Effects of Cyclin D1 Overexpression on the Expression of Other Genes Involved in Growth Control. In view of the above-described effects on growth control and cell cycle parameters, we examined the vector control and cyclin D1 overexpresser HC11 clones for expression, at the mRNA level, of three genes that play a critical role in growth control: c-*fos*, c-*myc*, and c-*jun*. As shown in Fig. 5, the expression of

Table 2 Flow cytometric analysis of cyclin D1-overexpressing derivatives of HC11 cells[a]

Cell line	G_0–G_1		S		G_2–M	
	% cells[b]	Time (h)[c]	% cells	Time (h)	% cells	Time (h)
HC11 - vt#3	28.7	4.0	57.8	8.1	13.4	2.0
HC11 - vt#4	23.0	3.2	65.0	9.2	12.0	1.7
Mean	25.9	3.6	61.4	8.7	12.7	1.9
HC11 - D1#5	41.2	6.7	45.2	7.4	13.6	2.2
HC11 - D1#7	55.5	8.6	35.0	5.4	9.5	1.2
HC11 - D1#19	61.5	9.2	30.1	4.5	9.5	1.2
HC11 - D1#20	49.7	7.4	39.4	5.9	10.9	1.6
Mean	52.0	8.0	37.4	5.8	10.9	1.6

[a] Exponentially growing cultures of the indicated cell lines were collected, and their DNA content was analyzed by flow cytometry. For additional details, see "Materials and Methods."
[b] The values indicate the percentage of the total cell population in the indicated phase of the cell cycle.
[c] The values indicate the length of each phase of the cell cycle based on the doubling time of the indicated cell line.

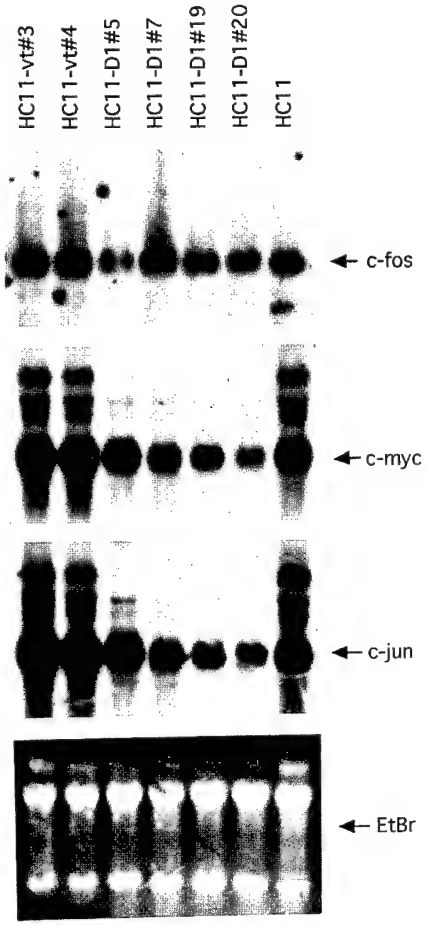

Fig. 5. Northern blot analysis of the immediate early genes, c-*fos*, c-*myc*, and c-*jun*. Ten μg of total RNA were subjected to agarose gel, transferred to a membrane, and hybridized with ^{32}P-labeled c-*fos*, c-*myc*, or c-*jun* probes. Representative ethidium bromide (*EtBr*) staining of the membrane is shown as a control for RNA loading. For additional details, see "Materials and Methods."

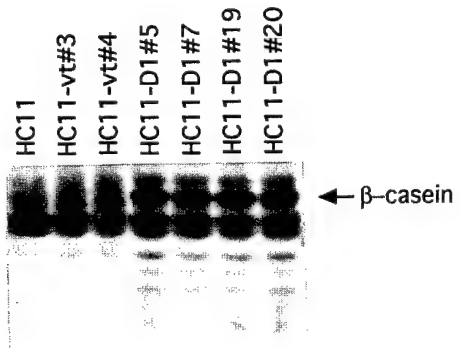

Fig. 6. Western blot analysis of protein extracts (50 μg) from the indicated cells, using an antibody to casein. *Arrow*, the β-casein protein band. For additional details, see "Materials and Methods."

c-*fos* was not altered by cyclin D1 overexpression. However, the expression levels of c-*myc* and c-*jun* were reduced. This is in contrast to our previous finding that c-*myc* and c-*jun* expression was not affected in derivatives of the human mammary epithelial cell line HBL 100 (51) or R6 rat fibroblasts (49) that overexpress cyclin D1.

Cyclin D1 Overexpression Induces Differentiation in Mouse HC11 Cells. Previous studies indicated that cyclin D1 is induced in HL-60 cells after treatment with the differentiating agents 12-*O*-tetradecanoyl phorbol-13-acetate, retinoic acid, or DMSO (59, 60). Because HC11 cells can be induced by growth hormones to secrete the milk protein β-casein (53–55), it was of interest to determine whether cyclin D1 overexpression altered the expression of this protein. Protein extracts were prepared from exponentially growing cells and examined by Western blot analysis with an anti-casein antibody. This antibody has been shown to detect casein proteins after induction of mammary differentiation in murine cells (54). We found increased expression of casein proteins in the cyclin D1-overexpressing clones (Fig. 6). Based on the known molecular mass (~30 kDa), β-casein protein was induced. Treatment of the overexpresser cells with a combination of prolactin, dexamethasone, and insulin, which induces casein expression in normal HC11 cells (53–55), did not further enhance the expression of casein proteins in the cyclin D1 overexpresser clones (data not shown). Thus, overexpression of cyclin D1 alone appears to induce differentiation in HC11 cells. For reasons that are not apparent, we could not reproducibly induce this β-casein protein in the

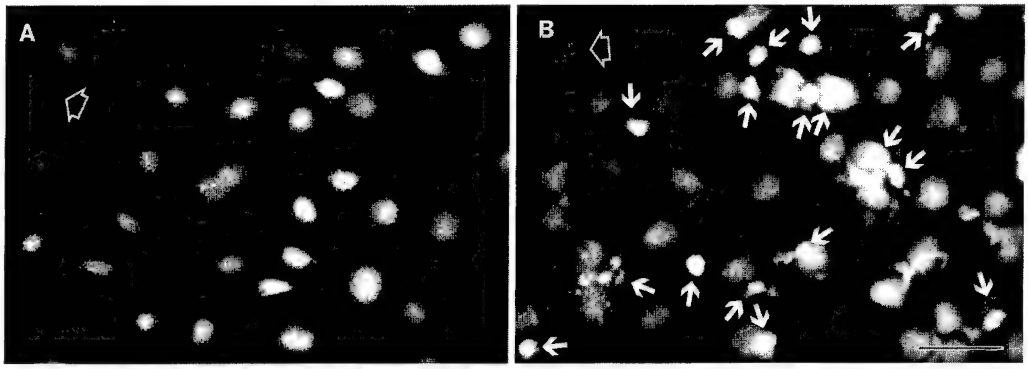

Fig. 7. Apoptosis in cyclin D1 overexpresser cells. Vector control HC11-vt#3 (*A*) and cyclin D1 overexpresser HC11-D1#19 (*B*) cells were maintained for 48 h in 0.1% FCS and then fixed and processed for immunocytochemistry, using FITC-conjugated wheat germ agglutinin to stain the cytoplasm and DAPI to stain the nucleus. Various types of evidence of apoptosis are indicated with *white arrows, i.e.,* contraction of the cell body, condensation of nuclear chromatin, and the production of apoptotic bodies. Normal nuclei are indicated with *dark arrows. A* and *B, bars,* 50 μm.

control cells with this combination of hormones (data not shown).

Overexpression of Cyclin D1 Induces Apoptosis. In previous studies, we have shown that stable overexpression of an exogenous cyclin D1 cDNA in the human mammary epithelial cell line HBL-100 inhibited growth (51). In the present study, cyclin D1 overexpression in the mouse mammary epithelial cell line HC11 also caused growth inhibition, although the effect was small (Table 1) compared to that seen in HBL-100 cells. Thus, in two different mammary epithelial cell lines, cyclin D1 overexpression inhibits rather than stimulates cell growth. It was of interest, therefore, to examine the possible effects of cyclin D1 overexpression on apoptosis. Apoptosis was determined by morphological criteria after staining the nuclei with DAPI. Cells undergoing apoptosis displayed contraction of the cell body, membrane blebbing, condensation of nuclear DNA (which caused bright DAPI staining), nuclear fragmentation, and the formation of apoptotic bodies (Fig. 7). When cells were grown in 10% FCS, less than 0.5% of the control HBL-100 and HC11 cells displayed apoptosis (Table 3). In contrast, about 2–4% of the HBL-100 and HC11 derivatives that overexpressed cyclin D1 displayed apoptosis. This effect was much more pronounced when cells were maintained in 0.1% serum for 72 h. Thus, with the overexpresser HBL-100 clone HBL-D1 #2, the apoptosis index was about 18, and with the four HC11 clones that overexpressed cyclin D1, the apoptotic index rose to between 34 and 75%. Thus, growth in 0.1% FCS caused about an 8-fold increase in the apoptotic index of the HBL-D1 #2 cells and as much as a 50-fold increase with the HC11 cyclin D1 overexpresser clones when compared to the results obtained with 10% FCS. On the other hand, growth of the control HBL-100 or HC11 cells in 0.1% FCS did not cause an appreciable increase in the apoptosis index (Table 3). Time course studies indicated that apoptosis was induced within 24 h after the withdrawal of 10% FCS and was at maximum at 48–72 h, and at all time points the extent of apoptosis was much greater in the cyclin D1 overproducers than in the control cells (data not shown). In view of the above results, it was of interest to see whether the mammary epithelial cell lines that overexpressed cyclin D1 were also more sensitive to the induction of apoptosis by various cytotoxic drugs when tested in the presence of 10% FCS. In recent studies from our laboratory,[4] we have found that the staurosporine derivative CGP 41251 (62), a potent and selective inhibitor of PKC, markedly induces apoptosis in human glioblastoma cell lines. When treated with 10 μM CGP 41251 for 48 h, the control HBL-100 and HC11 cells displayed some induction of apoptosis, but the apoptotic index was about 5–10-fold higher in the CGP 41251-treated cyclin D overexpresser derivatives of both of these cell lines (Table 4). A similar pattern was seen when these cell lines were treated with 1 μM CGP 41251, but the apoptotic indices were lower than those obtained with the 10 μM dose (Table 4). The induction of apoptosis by CGP 41251 in all of these cell lines was blocked by simultaneous treatment of the cells with

Table 3 Apoptosis index (%)

Indicated cell lines were plated in LabTek chamber slides. On the following day, cells were refed with either 10% FCS or 0.1% FCS and maintained for 72 h. After 72 h, cells in LabTek chamber slides were fixed and processed for determination of apoptosis (see Fig. 7). The values indicate the percentage of cells with an apoptotic morphology. For additional details, see "Materials and Methods."

Cell line	10% FCS	0.1% FCS
HBL-100	<0.5	3.9 ± 1.7
HBL-vt#1	<0.5	0.8 ± 0.4
HBL-D1#2	2.2 ± 1.7	18.0 ± 7.2
HC11	<0.5	<2.0
HC11-vt#3	<0.5	<2.0
HC11-vt#4	<0.5	<2.0
HC11-D1#5	1.7 ± 1.0	58.3 ± 8.9
HC11-D1#7	4.2 ± 3.3	34.4 ± 11.1
HC11-D1#19	2.2 ± 1.6	75.2 ± 8.4
HC11-D1#20	1.0 ± 0.9	50.9 ± 11.4

[4] M. Begemann, S. A. Kashimawo, Y. A. Choi, S. Kim, K. M. Christiansen, G. Duigou, M. Mueller, I. Schieren, S. Ghosh, D. Fabbro, N. M. Lompen, D. F. Heitjan, P. B. Schiff, J. N. Bruce, and I. B. Weinstein. Inhibition of the growth of glioblastomas by CGP 41251, an inhibitor of protein kinase C, and by a phorbol ester tumor promoter. Clinical Cancer Res., in press.

12-O-tetradecanoylphorbol-13-acetate (100 ng/ml), a potent activator of PKC (data not shown).

When compared to the HBL-vt#1 vector control cells, the HBL-D2 overexpresser cells also displayed a marked increase in apoptotic index when treated with the PKC inhibitors Ro31-8220 (Ref. 63; 1 μM) and calphostin C (Ref. 63; 0.1 μM) and the inhibitor of DNA synthesis hydroxyurea (0.1 mM; data not shown). Thus, overexpression of cyclin D1 in either a human or murine mammary epithelial cell line markedly sensitizes the cells to induction of apoptosis by certain agents, especially serum starvation, certain PKC inhibitors, and hydroxyurea.

Table 4 Apoptosis index (%)

All assays were done in complete growth medium with 10% FCS. Apoptosis was arrested 48 h after the indicated compounds were added. For additional details, see Table 3.

Cell line	Untreated	1 μM CGP41251	10 μM CGP41251
HBL-vt#1	<0.5	12.4	9.5
HBL-D1#2	7.0	26.4	43.2
HC11-vt#3	<0.5	1.0	5.0
HC11-D1#19	<2.0	6.6	43.8

Discussion

There is considerable evidence that cyclin D1 plays a critical role in cell cycle progression during the early to mid-G_1 phase of the cell cycle (1, 3, 4, 49, 50). The study of cyclin D1 is directly relevant to human breast cancer, because the *cyclin D1* gene is frequently amplified and/or overexpressed in both primary human breast carcinomas and breast carcinoma cell lines (30, 33, 34, 51, 64–70) and carcinogen-induced rat mammary carcinomas (71).

In studies in which an exogenous *cyclin D1* cDNA was overexpressed either stably or inducibly in murine, rat, or human fibroblasts, this led to shortening of the G_1 phase and, in some cases, enhanced growth and tumorigenicity (49–51, 72–74). To further understand the role of cyclin D1 in breast tumorigenesis, in previous studies we overexpressed cyclin D1 in the human mammary epithelial cell line HBL-100 (51). In contrast to the previous results obtained with fibroblasts, we found that cyclin D1 overexpression markedly inhibited the growth of the HBL-100 cells, and this was associated with lengthening of the S phase. Although the HBL-100 cells were originally isolated from normal human mammary epithelial cells, they express the SV40 large T-antigen and exhibit characteristics of transformation (75, 76). Therefore, we sought to overexpress cyclin D1 in a more normal mammary epithelial cell line. We chose the normal mouse mammary epithelial cell line HC11 because, although it is immortalized, it is highly epithelial, has normal growth properties, is nontumorigenic, and can be induced to express the milk protein casein upon stimulation with specific hormones (53–55). The same human cDNA construct used in our previous studies with rat fibroblasts and HBL-100 cells was introduced into the HC11 cells via retrovirus-mediated infection, and neo+ clones were selected, as described previously (49, 51). Several neo+ clones expressed about an 8-fold increase in cyclin D1 protein, as determined by Western blot analysis with a cyclin D1 antibody (Fig. 1). When compared to vector control clones, the cyclin D1 overexpressers had a similar exponential doubling time, but they had a lower saturation density and a lower cloning efficiency in soft agar assay, and there was an increased fraction of cells in the G_1 phase of the cell cycle (Tables 1 and 2). These results are in contrast to our previous findings in which stable overexpression of the same cyclin D1 construct in R6 rat fibroblasts stimulated growth and decreased the proportion of cells in the G_1 phase (49). In the human mammary epithelial cell line, HBL-100 overexpression of cyclin D1 inhibited growth and caused an increase in the proportion of cells in the S phase (51).

The growth-inhibitory effects of cyclin D1 are not confined to HBL-100 and HC11 cells, because in recent studies we found that when cyclin D1 was overexpressed in the normal human mammary epithelial MCF-10F cells, this also caused growth inhibition. To confirm that cyclin D1 overexpression can induce growth inhibition at the cell population level, we transfected the pRC/cytomegalovirus vector containing the *cyclin D1* cDNA into two normal human mammary epithelial cell lines (MCF-10F and 184B5). The transfected cells were selected in the presence of G418 for 2 weeks, and neo+ clones were stained and counted. Using this method, we found that the cyclin D1-transfected cells generated many fewer neo+ clones than cells transfected with vector alone. These studies provide further evidence that overexpression of cyclin D1 in mammary epithelial cells can inhibit rather than stimulate growth.

It is of interest that the HC11-cyclin D1 overexpresser cells expressed about a 3-fold increase in the level of expression of the cdkI protein $p27k^{kip1}$ (Fig. 3). We have also seen increased expression of the latter protein in derivatives of MCF-10F cells that overexpress cyclin D1 (data not shown). The increased levels of this inhibitory protein could account for the above-described growth inhibition and the increase of cells in the G_1 phase, because $p27^{kip1}$ can inhibit cyclin D1-cdk4, cyclin E-cdk2, and cyclin A-cdk2 kinase activities, to varying degrees (24–26). We suggest that in certain cell types, overexpression of cyclin D1 can induce the expression of $p27^{kip1}$, either directly or indirectly, perhaps because of a negative feedback regulatory loop involved in cell cycle control. It is of interest that overexpression of cyclin D1 in human fibroblasts is associated with increased expression of the cdkI protein $p21^{WAF1}$ (72). The high and unregulated level of cyclin D1 in the HC11 overexpresser clones might lead to overexpression of $p27^{kip1}$ and, therefore, to growth inhibition. However, the phenotype of these cells is quite complex; therefore, other explanations have not been excluded, especially because the HC11 cyclin D1 overexpresser cells displayed an increase rather than a decrease in *in vitro* cyclin D1-CDK4 kinase activity (Fig. 4*A*).

Overexpression of cyclin D1 in HC11 cells was also associated with increased levels of expression of the cyclin E and cyclin A proteins (Fig. 1). In addition to the characteristic 52 kDa cyclin E protein band, these cells also expressed two lower-molecular-mass (42 and 50 kDa) cyclin E-related proteins (Fig. 1). Similar lower-molecular-mass cyclin E proteins have been noted in a variety of human tumors (31, 32). In

addition to the increased levels of the major 58-kDa cyclin A protein, the HC11 cyclin D overexpresser cells also displayed a 56-kDa cyclin A-related protein (Fig. 1). To our knowledge, this form of cyclin A has not been described previously, and its precise nature is not known. Despite the increased levels of cyclins E and A, the HC11 cyclin D1 overexpresser cells did not display increased cdk2-associated *in vitro* kinase activity (Fig. 4*B*), perhaps because of the above-mentioned increased expression of $p27^{kip1}$.

A novel finding in the present study is that stable overexpression of human cyclin D1 in the HC11 cell line induces apoptosis, which was demonstrated by characteristic morphological criteria. We also observed the same phenomenon in a cyclin D1 overexpresser derivative of the human mammary epithelial cell line HBL-100. Thus, increased levels of a human cyclin D1 can induce apoptosis in both mouse and human mammary epithelial cells. Therefore, the effects observed with HC11 cells are not simply due to the expression of a human cyclin D1 in murine cells. It is of interest that increased expression of endogenous induction of cyclin D1 occurs in postmitotic neurons undergoing apoptosis (77) and that senescent fibroblasts also express increased levels of cyclin D1 (72, 78, 79). A recent study demonstrated that when HC11 cells were serum-starved for 48 h, there was induction of apoptosis, as determined by DNA ladder formation (80). However, using the same protocol, we have not been able to demonstrate DNA ladder formation in our control or cyclin D1 overexpresser HC11 cells, although the latter cells displayed striking morphological nuclear changes, characteristic of apoptosis (Figs. 6 and 7). This apparent discrepancy might be due to somewhat different experimental conditions. Other studies have shown that apoptosis can occur without internucleosomal DNA fragmentation and DNA ladder formation (81–86).

The difference in apoptotic indices between the HC11 and HBL-100 cyclin D1 overproducer cell lines and the corresponding vector control cell lines was particularly striking after serum starvation. Thus, when transferred from medium containing 10% FCS to medium containing 0.1% FCS, the cyclin D1 overexpresser cells displayed about a 8- to 24-fold increase in apoptosis when compared to similarly treated control cells (Table 3). These results are reminiscent of previous findings indicating that when fibroblasts that overexpress c-*myc* (87) or rho-p21 (88) are starved of serum, they also display increased apoptosis. Thus, the overexpression of genes involved in signal transduction and/or cell cycle control may, paradoxically, make these cells more dependent on growth factors for survival. We found that the cyclin D1 overexpresser cells were also much more sensitive to the induction of apoptosis by certain cytotoxic agents, including the PKC inhibitors CGP 41251, Ro31-8220, and calphostin C, and the inhibitor of DNA synthesis hydroxyurea. The effects of the PKC inhibitors are consistent with the apparent protective effect of PKC with respect to apoptosis in a variety of cell systems (89–92). Our findings may be relevant to the role of cyclin D1 in the *in vivo* physiology of the mammary gland, because cell death has been shown to play a pivotal role during the involution of the lactating mammary gland (93, 94) and in breast cancer (95, 96). We speculate that cyclin D1, in conjunction with other factors, regulates apoptosis in both normal and malignantly transformed mammary epithelium. In fact, apoptosis might, in unusual cases, account for the published cases of spontaneous regression of advanced breast cancer and other tumors (97, 98).

With respect to the molecular mechanism by which cyclin D1 regulates apoptosis, it is of interest that the proteins c-myc (87, 99), adenovirus E1A (100), human papillomavirus E7 (101), and the E2F transcription factor (102, 103) can induce apoptosis. All of these proteins can interact with the tumor suppressor protein Rb (104–108), which itself is required for survival of certain lineages throughout development (109, 110). However, apoptosis can also occur in the absence of the Rb protein (111, 112). Cyclin D1 is also known to bind to the Rb protein, and the cyclin D1-cdk4 complex can phosphorylate Rb (4, 113, 114). The murine mammary epithelial HC11 cells express the Rb protein (Fig. 4*C*), but this protein is not expressed in the human mammary epithelial HBL-100 cells (69, 70). Thus, it is not clear whether cyclin D1 enhances apoptosis in these two cell lines through its interactions with the Rb protein or through other mechanisms. Alternatively, free (unbound) cyclin D1 may target other genes that are involved in apoptosis. In fact, the activation of other kinases, *e.g.,* cdc2 (115) and PITSLRE (116), and increased expression of cyclin A (117) have been shown to correlate with apoptosis. Perhaps cyclin D1 overexpression leads to similar changes, thus resulting in apoptosis. In fact, we did observe increased expression of cyclin A and a novel lower-molecular-mass cyclin A-related protein in the HC11 cyclin D1 overproducers (Fig. 1). Although in some cell systems the tumor suppressor p53 can mediate apoptosis as a consequence of DNA damage or unbalanced cell growth (118), HC11 cells express a mutant form of p53 (119), and HBL-100 cells do not express detectable levels of this protein (69, 70). Therefore, it is unlikely that p53 plays a role in the effects on apoptosis found in the present studies. Finally, it is possible that the increased expression of $p27^{kip1}$ in the cyclin D1 overexpresser mammary epithelial cells plays a role in enhancing their sensitivity to the induction of apoptosis.

Regardless of the underlying mechanism, the results obtained with HC11 cells in the present study, taken together with our previous findings with HBL-100 cells (51), indicate that in both a murine and human mammary epithelial cell lines, stable overexpression of cyclin D1 can inhibit growth and enhance apoptosis. These findings are in striking contrast to the finding that overexpression of cyclin D1 in rodent fibroblasts is oncogenic, because it enhances growth and malignant cell transformation (49, 120, 121). Thus, the effects of increased expression of cyclin D1 in a particular cell type are highly context dependent. It is also paradoxical that increased expression of cyclin D1 in mammary epithelial cell cultures inhibits growth, because this gene is often amplified and/or overexpressed in both human (30, 33–34, 51, 64–70) and rat mammary (71) carcinomas. Presumably during the development of breast cancer, there are other events that compensate for these potential growth-inhibitory effects. Studies are in progress to identify the precise determinants

that influence the divergent responses of cells to increased expression of this important gene.

Materials and Methods

Cell Culture and Viral Transduction. The mouse mammary epithelial cell line HC11 was obtained from Dr. David Solomon and grown in RPMI 1640 plus 10% FCS, 10 ng/ml epidermal growth factor, and 5 μg/ml insulin, as described previously (53). The human mammary epithelial cell line HBL-100 was grown in McCoy 5A medium plus 10% FCS, as described previously (51, 61). Cell cultures were maintained in a 37°C incubator with 5% CO_2.

The construction of the cyclin D1 retroviral expression plasmid PMV7-CCND1 and the methods used for retrovirus packaging and transduction have been described previously (49). Briefly, viral supernatants were obtained after transfection of the PMV7 vector itself (control) or the PMV7-CCND1 construct into the amphotropic GPAM 12 packaging cell line. These viral supernatants were used to infect HC11 cells. Neo+ cells were selected in the presence of 700 μg/ml G418, and the drug-resistant colonies were picked and expanded for further analysis.

Protein Extraction and Western Blot Analysis. Protein extracts were prepared from exponentially growing cells, as described previously (51). Briefly, cells were collected, and the cell pellets were resuspended in lysis buffer [20 mM Tris-HCl (pH 7.4), 2 mM EGTA, 2 mM EDTA, 6 mM β-mercaptoethanol, 1% NP40, 0.1% SDS, and 10 mM NaF, plus the protease inhibitors aprotinin (10 μg/ml) and leupeptin (10 μg/ml), and 1 mM phenylmethylsulfonyl fluoride]. This suspension was sonicated three times with a Sonifier Cell Disruptor (Heat Systems-Ultrasonics, Plainview, NY). Protein concentrations were determined by the Bio-Rad assay method. For Western blotting, 50 μg of protein in the total cell lysates were subjected to 10% SDS-PAGE. The proteins on these gels were then transferred, using transfer buffer (25 mM Tris, 190 mM glycine, and 10% methanol), to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked with blocking buffer (50 mM Tris, 200 mM NaCl, 0.2% Triton X-100, and 3% BSA), and the membranes were then incubated with a 1:1000 to 1:2000 dilution of the indicated anti-human cyclin antibodies. The polyclonal antibodies to cyclin D1, E, A, cdk2, and cdk4 were obtained from UBI (Lake Placid, NY), and the monoclonal antibody to Rb was purchased from PharMingen (San Diego, CA). After washing with washing buffer (blocking buffer without 3% BSA), horseradish peroxidase-linked anti-rabbit donkey serum (1:5000; Amersham, Arlington Heights, IL) was added. Membranes were then washed with washing buffer, and immune detection was performed using the enhanced chemiluminescence Western blotting detection system (Amersham). The intensities of the cyclin D1 bands were quantitated by densitometric scanning.

***In Vitro* Assay for Cyclin D1- and cdk2-associated Kinase Activities.** cdk assays were performed as described previously (122). Briefly, for the cyclin D1-associated kinase assay, 150 μg of the cell extracts were immunoprecipitated with 2 μg of cyclin D1 antibody. One μg GST-Rb was incubated with each immunoprecipitate, plus 5 μCi [γ-^{32}P]ATP for 15 min at 30°C. The reaction mixture was then subjected to SDS-PAGE, and the GST-Rb phosphorylation was determined by autoradiography. For the cdk2 kinase assay, 25 μg of total cell extracts were used, and 2 μg of histone H1 were used as the substrate instead of GST-Rb.

Northern Blot Analysis. Northern blots were performed as described previously (51). Cells from exponentially dividing cultures were collected with a rubber policeman and washed three times with ice-cold PBS, lysed in lysis buffer (3 M LiCl and 6 M urea), and homogenized for 1 min with a PCU sonicator (Kinematica AG, Littau, Switzerland). The tubes were placed in ice overnight at 4°C. The next day, the tubes were centrifuged, and the pellets were resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) plus 0.5% SDS, followed by phenol/chloroform extraction. The RNA samples (10 μg) were electrophoresed in 1% agar-6% formaldehyde gels and blotted onto Hybond-N membranes (Amersham). The membranes were then preincubated in Church buffer at 65°C and then hybridized with ^{32}P-labeled probes to human cyclin D1, mouse D2 and D3, c-*fos*, c-*myc*, or c-*jun* for 16 h. The membranes were washed with 1× SSC (150 mM NaCl, 15 mM Na_3 citrate, pH 7.0) containing 0.2% SDS for 20 min at room temperature and then washed for 20 min at 65°C, using the same washing solution. After the final wash with 1× SSC at 65°C, the membranes were exposed to Kodak XAR-5 film with intensifying screens at −70°C.

Immunohistochemistry. Cells were grown on LabTek chamber slides that had been precoated with poly-D-lysine (0.1 mg/ml; Collaborative Research, Bedford, MA) in DMEM for 1 h. After the indicated exposures (Tables 3 and 4), the cells were fixed with 90% ethanol, 5% glacial acetic acid, and 5% H_2O for 30 min. The cell nuclei were first counterstained with DAPI (Boehringer-Mannheim, Indianapolis, IN) at a final concentration of 1.5 μg/ml in PBS for 30 min at room temperature and then visualized with fluorescence microscopy. In other experiments, cell bodies were stained with FITC-conjugated wheat germ agglutinin (Vector, Burlingame, CA), according to the manufacturer's instructions.

Flow Cytometric Analysis. Flow cytometric analysis was performed as described previously (51). Briefly, exponentially growing cultures of HC11 cells, or their derivatives, were trypsinized, collected, and washed twice with PBS. Cell pellets were fixed in 70% ethanol and stored at 4°C. On the day of the assay, the fixed cells were collected by centrifugation, and the pellets were resuspended in 0.8 ml containing 0.2 mg/ml propidium iodide, 0.6% NP40, and 1 mg/ml RNAse, and the suspension was incubated in the dark at room temperature for 30 min. The cell suspension was then filtered through a 60 μM Spectra mesh filter and analyzed on a Coulter EPICS 753 flow cytometer for DNA content. The percentage of cells in different phases of the cell cycle was determined with a ModFit 5.2 computer program.

Growth Curves and Saturation Densities. Growth curves, exponential doubling times, and saturation densities were determined as described previously (51). Cells (1×10^4/35-mm-diameter well) were plated in replicate six-well plates, in the presence of either 1 or 10% FCS. Every 2 days, aliquots of cells were counted for up to 13 days with a Coulter counter. Cells were refed with fresh medium every 2 days during this time course. The doubling times were calculated from the initial exponential phase of the growth curves, and the saturation densities were calculated from the plateaus of the growth curve.

Soft Agar Assay. Cells were grown in soft agar assays as described (56). These assays were carried out in 10% FCS. For the bottom layer of agar, 1 ml of 0.5% agar was placed in each 35-mm-diameter well of six-well plates. Then 2 ml of 0.3% top agar containing 1×10^5 cells were layered on top of the solidified layer of bottom agar. Colony formation was monitored by microscopy for up to 14 days, and the final numbers of colonies per well that were >0.1 mm in diameter were determined.

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Overexpression of Cyclin E in the HC11 Mouse Mammary Epithelial Cell Line Is Associated with Growth Inhibition and Increased Expression of $p27^{Kip1}$[1]

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ABSTRACT

To elucidate the role of cyclin E in cell growth and tumorigenesis in mammary epithelial cells, we have used retrovirus-mediated transduction to generate derivatives of the nontransformed HC11 mouse mammary epithelial cell line that stably express a human cyclin E cDNA (HU4). These derivatives expressed two distinct forms of the exogenous cyclin E protein, which were about M_r 50,000 and M_r 42,000, thus corresponding to endogenous cyclin E proteins found in human cells. In contrast to results obtained previously in fibroblasts, overexpression of the HU4 cyclin E cDNA in HC11 cells was associated with an increase in cell size, lengthening of G_1, and inhibition of both anchorage-dependent and -independent growth. Furthermore, when quiescent serum-starved cells were restimulated with serum, entry into the S-phase was delayed in the overexpressor cells. Under these conditions, there was also delayed induction in the expression of the endogenous cyclin E protein and in other events involved in the G_1 transition. Despite the high level of expression of the exogenous cyclin E, the derivatives did not display increased cyclin E-associated *in vitro* kinase activity. The HC11 cells that overexpressed the exogenous cyclin E displayed an increase in the cyclin/cyclin-dependent kinase inhibitor $p27^{Kip1}$ in both asynchronous exponentially dividing and synchronous cell populations. These findings indicate that increased expression of this cyclin E cDNA in HC11 cells inhibits rather than stimulates growth and that this may be due to increased expression of the inhibitor $p27^{Kip1}$.

INTRODUCTION

Cyclins are a family of genes involved in the regulation of cell cycle progression in eukaryotes, through mechanisms that have been highly conserved during evolution (1–3). Originally described in marine invertebrates as proteins that displayed a remarkable periodicity in abundance during the cell cycle, they were subsequently identified in all eukaryotic cells from yeast to humans. They function essentially by controlling the timing of activation and the substrate specificity of a series of Cdks,[4] which are sequentially activated during the cell cycle. Several cyclins and Cdks have been identified in mammalian cells. Specific cyclins bind to specific Cdks, thus activating their kinase activity. Each of these cyclin/Cdk complexes is activated at a specific point during the cell cycle and has a specific set of substrates (3–6).

G1 cyclins regulate the progression of cells through G_1 and drive entry into S phase. Three D-type cyclins, D1, D2, and D3, act at mid-G_1 by complexing with either Cdk4 or Cdk6 (1, 3). Cyclin E acts in late G_1 by complexing with Cdk2 (7, 8). Several types of evidence indicate a rate-limiting role for these cyclins in G_1 progression. Thus, overexpression of D-type or E cyclins accelerates the G_1 to S transition and decreases cell size (9–13). On the other hand, antibodies to cyclin D1 (14, 15), cyclin D2 (16), or cyclin E (17) inhibit entry into S phase when injected into cells during the G_1 phase of the cell cycle. However, their functions are not redundant (18). Thus, injection of antibodies, or treatment with antisense oligonucleotides to cyclin D1, blocks entry into S phase in cells with wild-type pRb but not in cells lacking pRb function (19, 20). In contrast, injection of anti-cyclin E antibodies arrests cells in G_1, even in Rb− cells (17). Moreover, the cyclin D1/Cdk4 complex can phosphorylate *in vitro* the pRb-related protein p107 but not the pRb-related p130 (Rb2), whereas the contrary is true for the cyclin E/Cdk2 complex (21, 22).

Cyclin E is a nuclear protein originally isolated by screening human cDNA libraries for genes that could complement the loss of G1 cyclins in *Saccharomyces cerevisiae* (23, 24). As mentioned above, expression of cyclin E is cell cycle regulated and rises to a maximum level in late G_1, after the increase in cyclins D1 and D2, and it associates with and activates Cdk2 (8, 25). The cyclin E/Cdk2 complex shows strong kinase activity shortly before cells enter S phase and leads to further phosphorylation of the pRb protein (26–30). The activity of the cyclin E/Cdk2 complex is further regulated by members of a family of CDIs, which include $p21^{Waf1}$ (also designated Cip1, Pic1, Sd1, and Cap20) and $p27^{Kip1}$ (also called Ick and Pic2) (reviewed in Refs. 2 and 31–33).

Since cyclins play a pivotal role in controlling the order of events in the cell cycle, perturbations in their activity can have dramatic effects on cell cycle control and cell proliferation. Several lines of evidence indicate an involvement of cyclins and other cyclin-related genes in cancer. Most of this evidence relates to cyclin D1. This gene is frequently amplified and/or overexpressed in several types of human tumors (34–36). In contrast, the cyclin E gene is only rarely amplified in tumor cells (34, 37, 38). However, expression of the cyclin E protein is deregulated in several human cancers (39, 40). Thus, increased expression of multiple cyclin E-related proteins has been reported in human cancer cell lines and several types of primary tumors (37, 40). In breast cancer, these alterations in cyclin E expression have been associated with the stage and grade of the tumors (40).

To further address the role of cyclin E in growth control and tumorigenesis in mammary epithelial cells, we have used retrovirus-mediated transduction to generate derivatives of the nontransformed HC11 mouse mammary epithelial cell line (41) that stably overexpress a human cyclin E cDNA (HU4) originally isolated from a glioblastoma cell line (23). It has been reported that stable overexpression of the same cDNA in rat and human fibroblasts induced a decrease in cell size, shortened G_1, and reduced the requirement of serum for growth (10). The present study demonstrates that overexpression of the same cyclin E cDNA in HC11 cells inhibited, rather than stimulated, the growth of these cells. There was also an increase in the percentage of cells in G_1 and a lengthening of the G_0-to-S transition. We show that these effects were associated with an increase in the level of expression of the CDI $p27^{Kip1}$. The implications of these findings are discussed.

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[4] The abbreviations used are: Cdk, cyclin-dependent kinase; CDI, Cdk inhibitory protein; FBS, fetal bovine serum; EGF, epidermal growth factor; GM, growth medium; FACS, fluorescence-activated cell sorter.

MATERIALS AND METHODS

Cell Culture. The HC11 mouse mammary epithelial cell line was clonally derived from a spontaneously immortalized mammary epithelial cell culture originally established from a midterm-pregnant BALB/c mouse (41). HC11 cells were grown and maintained in RPMI 1640 supplemented with 8% FBS, 10 ng/ml murine EGF, and 5 μg/ml bovine insulin (GM). For some experiments, cells were grown in the absence of these additional growth factors. The medium used to differentiate the cells contained RPMI 1640, 8% FBS, 1 μM dexamethasone, 5 μg/ml insulin, and 5 μg/ml bovine prolactin (DIP medium). HC11 cells and derivatives were induced to produce β-casein by growing them, and maintaining them for 2 days at confluence, in GM. These "competent" cultures were then incubated for 2 days in the DIP medium. For cell synchronization studies, the cells were plated in GM at a density of 2×10^6 cells per 15-cm-diameter plate and cultured for 24–36 h. Then, after washing twice with RPMI 1640, the cells were incubated in RPMI, with no serum or growth factors, and cultured for an additional 72 h. The normal human breast and human breast cancer cell lines used in Fig. 8 were obtained from the American Type Culture Collection and cultured as recommended by the supplier. The human immortalized mammary epithelial cells 184B5 and 184A1 (a gift from M. Stampfer, Lawrence Berkeley Laboratory, Berkeley, CA) were grown in supplemented MCDB 170 medium (Clonetics Corporation, San Diego, CA), as described previously (42). Rat-1 cells were obtained from J. M. Roberts (Fred Hutchinson Cancer Center, Seattle, WA) and cultured in DMEM medium plus 10% calf serum.

Construction of Retrovirus Vectors and Viral Transduction. The full-length human cyclin E cDNA (HU4), originally isolated from a human glioblastoma cell line (23), was subcloned into the *Hin*dIII site of the retroviral vector PMV12 polylinker (PMV12pl; Ref. 43) in the sense orientation. To prepare infectious retrovirus particles, the resulting PMV12-cycE plasmid, or the control vector PMV12pl, were transfected into the Y2 ecotropic retrovirus packaging cell line (44) by the calcium phosphate precipitation method, as described previously (45). The transfected cells were selected by growth in 400 μg/ml of hygromycin (Boehringer Mannheim Corp., Indianapolis, IN) for 15 days. The hygromycin-resistant clones were then pooled and expanded. The viral supernatants, containing defective recombinant retrovirus particles carrying the respective sequences, were harvested, filtered, and then used to infect HC11 cells. The HC11 cells were infected with the retrovirus as follows. The cells were seeded at a density of 5×10^5 per 10-cm dish in GM. After 24 h, they were infected with 3 ml of medium containing serial dilutions of the viral stock solution and 8 μg/ml of Polybrene (Sigma Chemical Co., St. Louis, MO). After 4 h, 7 ml of fresh GM medium were added to each plate. Twenty four h later, the medium was replaced with fresh GM medium. After two days, the cells were trypsinized and replated into GM plus 600 μg/ml hygromycin (selection medium). Following selection for cells resistant to hygromycin (hph), several individual resistant clones were randomly isolated, both from the cultures infected with the PMV12-cycE construct and the cultures infected with the PMV12pl vector. The cultures were then expanded and frozen as seed stocks. Resistant clones were grown in GM containing 300 μg/ml hygromycin. After 4–6 weeks in continuous culture, each clone was replaced from frozen seed stocks, since with serial passage they tended to lose expression of the exogenous cyclin E.

Growth Studies. The exponential doubling times and saturation densities were determined essentially as described previously (46). Cells were plated at a density of 1×10^4 cells per 35-mm-diameter well in triplicate. The number of cells per well was determined every 2 days for the subsequent 14 days, using a Coulter counter. Cells were refed with fresh medium every 2 days during this time. The doubling times were calculated from the initial exponential phase of the growth curves and the saturation densities from the plateau of the growth curves. Growth in soft-agar was also performed as described previously (46). For the bottom layer of agar, 1 ml of 0.5% agar in GM was placed in each 35-mm well of 6-well plates. Then 2 ml of 0.3% agar in GM containing 1×10^5 cells were layered on top of the solidified layer of bottom agar. Colony formation was monitored by microscopy for up to 14 days, and the final numbers of colonies larger than 0.1-mm diameter were determined.

Flow Cytometric Analysis. Cells were trypsinized, collected, and washed twice with PBS. Cell pellets were resuspended in 1 ml PBS and fixed in 5 ml of 70% ethanol and stored at 4°C. On the day of the analysis, cells were collected by centrifugation, and the pellets were resuspended in 0.2 mg/ml of propidium iodide in HBSS containing 0.6% NP40. RNase (1 mg/ml; Boehringer Mannheim) was added, and the suspension was incubated in the dark at room temperature for 30 min. The cell suspension was then filtered through a 41 μM Spectra mesh filter (Spectrum, Houston, TX) and analyzed for DNA content on a Coulter EPICS 753 flow cytometer. The percentage of cells in different phases of the cell cycle was determined using a ModFit 5.2 computer program.

DNA Synthesis. Cells were plated in triplicate in 24-well plates at a density of 2×10^4 cells/well and incubated 24–36 h in GM medium. They were then rinsed twice with RPMI 1640 and grown in RPMI 1640 without serum or growth factors. After 72 h (time 0), complete GM was added to the cultures. At the indicated time points, the cultures were labeled for 1 h with [^{3}H]thymidine (1 μCi/ml) (Amersham, Arlington Heights, IL) and then washed with ice-cold PBS and extracted with 10% cold trichloroacetic acid for 15 min on ice. After solubilization in 0.5 N NaOH, trichloroacetic acid-insoluble radioactivity was determined by liquid scintillation counting.

Northern Blot Analysis. Cells from exponentially growing cultures were collected with a rubber policeman into 50-ml tubes, washed three times with ice-cold PBS, and then lysed by suspension in lysis buffer (3 M LiCl and 6 M urea) and homogenized for 1 min using a Polytron sonicator (Brinkmann Instruments, Westbury, NY). The tubes were kept on ice overnight at 4°C. After centrifugation, the pellets were resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) plus 0.5% SDS and extracted with phenol/chloroform. The RNA samples (10 μg) were electrophoresed in 1% agar-6% formaldehyde gels and blotted onto Hybond-N membranes (Amersham). The blotted membranes were preincubated in Church buffer (47) at 65°C and then hybridized with ^{32}P-labeled probes to human cyclin E, cyclin D1, or cyclin A for 16 h. The membranes were washed with 1× SSC (150 mM sodium chloride, 15 mM sodium citrate) containing 0.2% SDS for 20 min at room temperature, followed by 20 min at 65°C. After the final wash with 1× SSC at room temperature, the membranes were exposed to Kodak XAR-5 film with intensifying screens at −70°C.

Immunoreagents. The polyclonal antibodies to cyclin D1, E, and A and to Cdk2 and Cdk4 were obtained from Upstate Biotechnology (Lake Placid, NY). The monoclonal antibodies to cyclin E (clone HE12) and to pRb (clone G3-245) were purchased from PharMingen (San Diego, CA). The polyclonal antibodies to $p21^{Waf1}$ and $p27^{Kip1}$ were from Santa Cruz Biotechnology (Santa Cruz, CA).

Immune Complex Kinase Assay. Cdk enzyme assays were performed as described previously (48) with minor modifications. Cells were resuspended in kinase-lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1% Tween 20, 10% glycerol, 0.1 mM phenylethylsulfonyl fluoride, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin; all of these chemicals were from Sigma] and sonicated two times using a Sonifier Cell Disruptor (Ultrasonics, Inc., Plainview, NY). After centrifugation, clarified materials (50 μg) were incubated with protein A-Sepharose for 1 h at 4°C for preclearing in IP buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, and 0.1% Tween 20]. Immunoprecipitations were carried out with 2 μg of the indicated antibody, and immunocomplexes were recovered with protein A-Sepharose. For H1 kinase activity, the protein A beads were washed four times with IP buffer, twice with washing buffer [50 mM HEPES (pH 7.5) and 1 mM DTT], and once with kinase buffer [50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM NaF, and 0.1 mM sodium orthovanadate]. The final pellet was resuspended in 30 μl of kinase buffer supplemented with 2 μg of Histone H1 (Boehringer Mannheim) and 5 μCi of [γ-^{32}P]ATP (Amersham) and incubated for 15 min at 30°C. The reaction was stopped by the addition of 25 μl 2×-concentrated Laemmli sample buffer. The samples were separated on a SDS-10% polyacrylamide gel, and the phosphorylated histone H1 was visualized by autoradiography. For lysate mixing assays, lysates (50 μg) of cells collected at different time points after releasing from serum starvation were boiled for 5 min, and the precipitated proteins were cleared by centrifugation. The remaining supernatants were mixed with 50 μg of a cell lysate extracted from HC11-V#1 cells at 20 h after releasing them from serum starvation. The mixed lysates were immunoprecipitated with polyclonal anti-cyclin E antibody and assayed for H1 kinase assay, as described above.

Immunoblotting and Immunoprecipitation. Protein extraction and immunoprecipitation were performed as described above for the H1 kinase assay.

Proteins from total cell lysates (50 μg) or immunoprecipitates (see above) were used for Western blot analysis, as described previously (46). Samples were electrophoresed by SDS-PAGE and then transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). Blots were then incubated with blocking buffer (50 mM Tris, 200 mM NaCl, 0.2% Triton X-100, and 3% BSA) for 90 min at room temperature. Different dilutions were used for different primary antibodies. Immunodetection was performed using the enhanced chemiluminescence kit for Western blotting detection (Amersham).

To detect the exogenous human cyclin E in cyclin E/Cdk2 complexes, 50 to 100 μg of cell lysates were immunoprecipitated with antiserum to one subunit (*i.e.,* anti-Cdk2) and blotted with the monoclonal anti-cyclin E antibody. For the experiment shown in Fig. 2*B*, the polyclonal anti-cyclin E antibody was used for immunoprecipitation.

Immunohistochemistry. Cells cultured on glass slides were washed twice with PBS, fixed with cold methanol for 3 min at −20°C, and permeabilized with 0.5% NP40 in PBS for 5 min at room temperature. After blocking with goat or horse serum for 1 h at room temperature, cells were incubated with the polyclonal or the monoclonal anti-cyclin E antibody, respectively, overnight at 4°C (1 μg/ml in PBS with 10% serum). After washing, immunostaining was performed using mouse IgG or rabbit IgG Vectastain ABC kits and diaminobenzidine (Vector Laboratories), as described (36). As a control, the slides were stained without the primary antibody to monitor background staining.

Statistical Analysis. Analyses were performed using Student's *t*-test.

RESULTS

Generation of Derivatives of the HC11 Mouse Mammary Epithelial Cell Line That Stably Overexpress Cyclin E. To address the role of cyclin E in cell cycle control and transformation in mammary cells, we overexpressed a human cyclin E cDNA in the nontransformed mouse mammary epithelial cell line HC11. This cell line has been used extensively for studying the roles of different genes in mammary tumorigenesis (49, 50). A full-length human cyclin E cDNA (HU4), originally isolated from a human glioblastoma cell line (23), was subcloned into the *Hin*dIII site of the retroviral vector PMV12-polylinker (PMV12pl) in the sense orientation. The resulting PMV12-cycE plasmid or the control vector PMV12pl was transfected into the Y2 ecotropic retrovirus packaging cell line (44) by the calcium phosphate precipitation technique, as described previously (45). The viral supernatants, containing defective recombinant Moloney murine leukemia viruses carrying the respective sequences, were harvested, filtered, and then used to infect HC11 cells. Following selection for resistance to hygromycin (hph), several resistant clones were isolated, both from the cultures infected with the PMV12-cycE construct and the cultures infected with the PMV12pl vector (vector control cells; see "Materials and Methods" for details). In multiple transduction studies, the number of hph^+ clones obtained with the PMV12-cycE construct was much fewer (only about 50%) than the number obtained with the control PMV12pl construct (data not shown). This finding suggested that overexpression of this cyclin E cDNA was toxic to many of the HC11 derivatives. Individual resistant clones were chosen for subsequent studies, rather than pools, since when pools of resistant clones were examined, we observed a progressive loss of cyclin E overexpression during serial passages (data not shown). This finding is consistent with the growth-inhibitory effects described below.

Expression of the exogenous cyclin E gene was verified by Northern blot hybridization and also by Western blot analysis using either a polyclonal anti-cyclin E antibody or a specific anti-human cyclin E monoclonal antibody. Fourteen of 18 clones examined showed high expression of the exogenous cyclin E and the corresponding mRNA. As shown in Fig. 1*A*, the level of the 5′-LTR-cyclin E-tk*hph*-LTR-3′ transcript was very high in the cyclin E-overexpressing clones compared to the level of the endogenous cyclin E mRNA in the vector control cells. The latter mRNA could be detected only after a longer exposure to the X-ray film.

The polyclonal anti-cyclin E antibody recognized at least two distinct forms of endogenous cyclin E in the exponentially growing parental and vector control HC11 cells, a major band at about M_r 55,000 and a minor band at about M_r 50,000. Using the same antibody in the cyclin E-overexpressing cells, we found a marked increase in the level of the M_r 50,000 cyclin E band. In a previous study (10), introduction of the same cyclin E cDNA into fibroblasts also led to the expression of a M_r 50,000 protein. With the polyclonal antibody, we also detected a faint M_r 42,000 band in some of the overexpressor clones. However, when a specific antihuman cyclin E monoclonal antibody was used, a prominent M_r 42,000 band was detected in all of the overexpressor clones, and the levels of this protein varied with the level of the M_r 50,000 band (Fig. 1*B*). Unless otherwise indicated, in subsequent studies we used the polyclonal anti-cyclin E antibody since it enabled us to detect both the exogenous and the endogenous cyclin E proteins without losing any information, and the level of the M_r 50,000 band also reflected the level of the M_r 42,000 band.

To evaluate the subcellular localization of the overexpressed cyclin E protein, immunostaining for cyclin E was performed on both the control and cyclin E-overexpressing cell lines. The staining was almost exclusively nuclear in all of the cell lines examined, and the intensity was markedly increased in the cyclin E-overexpressing cells (Fig. 1*C*).

Effects of Cyclin E Overexpression on Cell Cycle Parameters and Cell Growth. To evaluate the phenotypic effects of cyclin E overexpression in HC11 cells, several parameters were examined, in parallel, in the parental cell line (HC11), two vector control cell lines (HC11-V#1 and #2), and five cyclin E overexpressor clones (HC11-E#2, #8, #10, #12, and #13) that expressed various levels of the exogenous cyclin E (Fig. 1*B*).

The HC11-cyclin E clones did not display any morphological evidence of malignant transformation, and they did not form transformed foci. They were, however, morphologically different from the control cells since they were somewhat larger in diameter and showed cytoplasmic elongations, in both low- and high-density cultures, which were only rarely seen in the control cells (data not shown). When the cells were analyzed by forward angle light scatter, the results were consistent with an increase in cell size when compared to the control cells (Table 1).

The effects of increased expression of cyclin E on cell cycle distribution were analyzed by FACS analysis of exponentially growing cell cultures (Table 1). The cyclin E-overexpressing clones showed an increase in the percentage of cells in G_1 and a reduction of cells in the S phase. Although these changes were not dramatic, they were reproducible and statistically significant ($P < 0.01$). The results shown in Table 1 were reproducible in more than 10 independent experiments.

We then compared the growth curves of monolayer cultures of the cyclin E-overexpressing clones and control cells. The overexpressing clones displayed a longer exponential doubling time than the control cells (about 18 h *versus* 15 h; Table 2). These differences, although not striking, were also reproduced in several experiments. Furthermore, there was about a 2-fold decrease in the saturation densities of the cyclin E-overexpressing clones when compared to the control cells (Table 2). Similar changes were also observed when cells were grown in medium containing only FBS (data not shown). Nor was there any evidence that the overexpression of cyclin E in HC11 cells decreased the requirement for growth factors since, when either the control or cyclin E overexpressor cells were starved of serum, >90% of the cells arrested in the G_0-G_1 phase (data not shown).

The same cell lines were also assayed for anchorage-independent

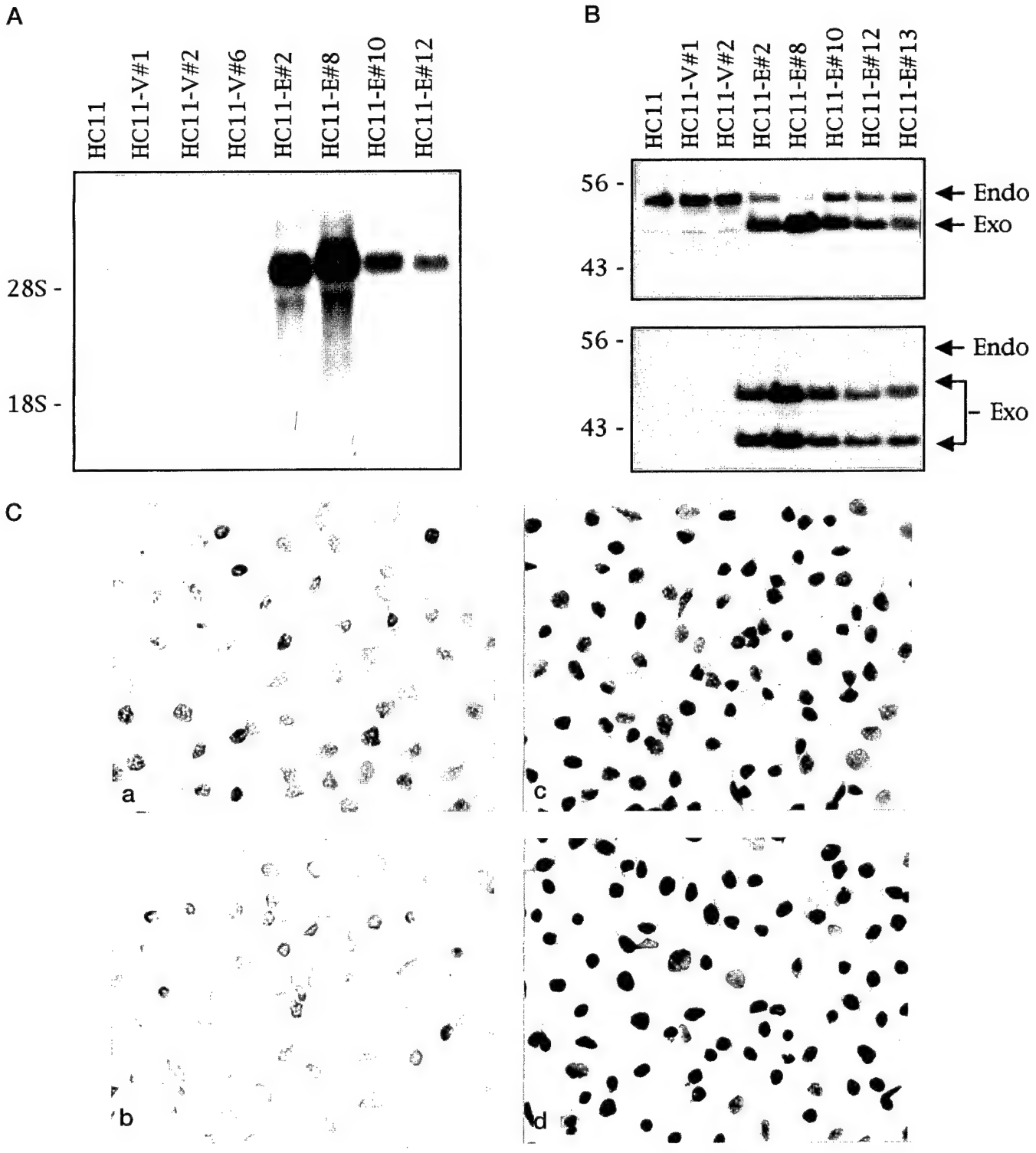

Fig. 1. Constitutive overexpression of human cyclin E in the HC11 mammary epithelial cell line. *A,* Northern blot analysis of RNA from the parental HC11 cell line, three vector control cell lines (HC11-V#1, -V#2, and -V#6), and four cyclin E-overexpressing cell lines (HC11-E#2, -E#8, -E#10, and -E#12). Total RNA (10 μg) from the indicated cell lines was examined by Northern blot analysis. A [^{32}P]dCTP-labeled human cyclin E cDNA was used as the probe. *Left,* the position of the 5.0-kb (28S) and 2.0-kb (18S) RNAs. *B,* Western blot analysis of cyclin E protein. Whole-cell lysates were extracted from exponentially growing cultures of the indicated cell lines. Fifty μg of proteins were resolved by 10% SDS-PAGE and transferred to an Immobilon membrane. Duplicate blots were probed with polyclonal anti-cyclin E (*top panel*) and a monoclonal antihuman cyclin E (*bottom panel*) antibodies, and immunoreactive bands were detected by enhanced chemiluminescence. *Left,* molecular size markers in kilodaltons. *Right,* the positions of the endogenous (*Endo*) and the exogenous (*Exo*) cyclin E proteins. *C,* nuclear localization of both endogenous and exogenous cyclin E proteins. Asynchronous exponentially growing HC11-V#1 vector control (*a* and *b*) and HC11-E#8 cyclin E overexpressor cells (*c* and *d*) were analyzed for cyclin E expression using the polyclonal (*a* and *c*) or the monoclonal (*b* and *d*) anti-cyclin E antibodies. The absence of reactivity in the vector control cells with the monoclonal antihuman cyclin E antibody (*b*) confirms the specificity of the staining. For additional details, see "Materials and Methods."

Table 1 *Flow cytometric analysis of cyclin E-overexpressing derivatives of the HC11 mouse mammary cell line*[a]

Cell line	Cell cycle distribution			Mean cell size[b]
	G_0–G_1[c]	S	G_2–M	
HC11	32.8	57.5	9.7	92.9
HC11-V#1	28.9	53.7	17.4	95.1
HC11-V#2	23.7	56.3	20.0	92.1
HC11-E#2	38.7	50.2	11.1	100.1
HC11-E#8	43.5	45.9	10.6	100.7
HC11-E#10	49.2	38.8	12.0	94.7
HC11-E#12	56.4	34.8	8.8	98.9
HC11-E#13	56.5	26.5	17.0	93.5

[a] Exponentially growing cultures of the indicated cell lines were collected, and the DNA content was analyzed by flow cytometry.

[b] The numbers given for the mean cell size represent the mean values of forward angle light scatter for 10,000 cells.

[c] The values indicate the percentage of the total cell population in the indicated phase of the cell cycle. The percentage of cells in G_1 was significantly different between vector control and cyclin E overexpressor cells ($P < 0.01$).

Table 2 *Growth properties of the cyclin E-overexpressing derivatives of the HC11 mouse mammary cell line*

Cell line	Growth in monolayer culture		Growth in soft agar
	Doubling time (h)[a]	Saturation density ($\times 10^6$)[b]	Colony-forming efficiency (%) Size >0.05 mm
HC11	16.4	8.7	0.84
HC11-V#1	14.6	11.4	1.66
HC11-V#2	15.3	11.0	3.57
HC11-E#2	17.5	4.7	0.05
HC11-E#8	18.0	6.6	0.09
HC11-E#10	17.2	4.8	0.24
HC11-E#12	19.7	5.9	0.00
HC11-E#13	18.3	4.2	0.09

[a] The doubling time corresponds to the initial exponential phase of cell growth (see "Materials and Methods" for details). The doubling time was significantly longer in the cyclin E overexpressor than in the vector control cells ($P < 0.01$).

[b] The numbers given for the saturation densities represent the total number of cells per 35-mm well when the cultures reached a plateau. The difference in the saturation densities between vector control and cyclin E overexpressor cells was also significant ($P < 0.01$).

growth. Consistent with previous studies (50), the parental HC11 cells and the vector control cells were able to form colonies in agar but with a low cloning efficiency (1–2%). However, the colonies were quite large (0.1–0.3-mm diameter). With the cyclin E-overexpressing clones, the colony-forming efficiency was only about 0.1% (Table 2), and most of the colonies were less than 0.1-mm in diameter. These differences were also highly reproducible.

The above results were unexpected in view of previous evidence that the level of cyclin E is a rate-limiting event for G_1 progression in mammalian cells (10, 12, 17, 18). Moreover, a previous report indicated that when the same cyclin E cDNA (HU4) was stably overexpressed in either rodent or human fibroblasts, it caused shortening of G_1, a decrease in cell size, and a decreased growth requirement for serum (10). The latter changes were associated with an increase in the amount of cyclin E-associated histone H1 kinase activity in exponentially growing cells (10). Therefore, we carried out similar kinase assays with the HC11 cells. We found that when exponentially growing cells were analyzed for the amount of cyclin E-associated kinase activity, no significant increase was observed between the cyclin E-overexpressing cells and the control cells (Fig. 2*A*). Similar results were also obtained for Cdk2- and cyclin A-associated kinase activity (data not shown). To exclude the possibility that these results were due to inability of the polyclonal anti-cyclin E antibody used in this assay to immunoprecipitate the exogenous cyclin E, cell extracts from exponentially growing cells were immunoprecipitated with the polyclonal anti-cyclin E antibody, separated on SDS-PAGE, and analyzed by immunoblotting with the monoclonal antihuman cyclin E antibody. As shown in Fig. 2*B*, the exogenous M_r 50,000 and M_r 42,000 cyclin E bands were readily detectable in the immunoprecipitates from the HC11-cyclin E clones. Confirmatory results were obtained by immunoprecipitation of [^{35}S]methionine-labeled cell extracts with the same antibody (data not shown).

The above described effects on cell growth and cell cycle parameters prompted us to analyze whether cyclin E overexpression in HC11 cells had a significant effect on the expression of other important cell cycle-related proteins. When exponentially proliferating cultures of the HC11-cyclin E derivatives were examined by immunoblotting, we could not detect any significant changes in the levels of expression of Cdk2, Cdk4, cyclin D1, cyclin A, proliferating cell nuclear antigen, or pRb proteins when compared to control cells (data not shown). The expression of cyclin D1 and cyclin A was also analyzed at the mRNA level, and again no significant differences could be detected between the cyclin E overexpressor clones and control cells (data not shown).

Effects of Cyclin E Overexpression on the G_0-to-S Transition. To further explore the effects of cyclin E overexpression on cell cycle parameters, we next used synchronized cell populations to analyze the interval from G_0 to S phase in the cyclin E-overexpressing derivatives and control cells. The HC11-cyclin E clones and control clones were synchronized by serum starvation, which caused accumulation of more than 90% of both types of cells in G_0-G_1, as assessed by FACS analysis (data not shown). FACS analysis of these cells at different time points after refeeding with complete growth medium showed that the parental cell line and the control clones entered the S phase at about 12 h after serum restimulation, whereas the clones that overexpressed cyclin E entered the S phase at about 15 h or later (data not shown). A similar difference was seen when the initiation of DNA synthesis was measured by [^{3}H]thymidine incorporation (Fig. 3*C*). This difference was consistently observed for several cyclin E-overexpressing clones and in repeated studies (data not shown). The G_0-to-S progression was also slower for the cyclin E-overexpressing derivatives than the control cells when serum starved cells were stimulated to enter the cell cycle by refeeding them with medium containing only EGF (data not shown).

Total cell extracts were collected at different times after serum stimulation of quiescent cultures and analyzed by immunoblotting for the expression of G1 cyclins and Cdks. We found that in the parental

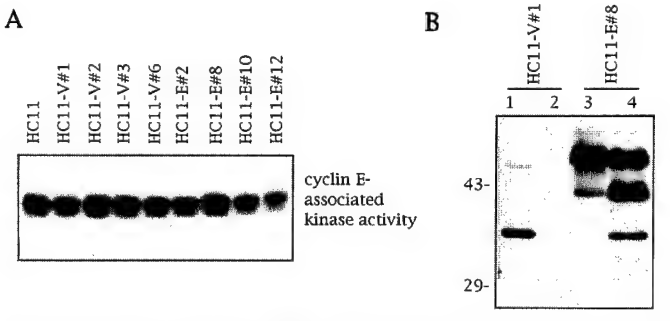

Fig. 2. Effects of cyclin E overexpression on kinase activity. *A*, 50 μg of whole-cell lysates from exponentially growing cultures of the indicated cell lines were used to analyze the histone H1 kinase activity in anti-cyclin E immunoprecipitates. The polyclonal anti-cyclin E antibody was used for the assay. *B*, ability of the polyclonal anti-cyclin E antibody to immunoprecipitate the exogenous cyclin E. Fifty μg of whole-cell lysates from the HC11-V#1 vector control cell line (*Lane 2*) and from the HC11-E#8 cyclin E-overexpressing cell line (*Lane 3*) were immunoprecipitated with the polyclonal anti-cyclin E antibody. Immunocomplexes were separated on 10% SDS-PAGE, transferred to an Immobilon membrane, and immunoblotted with the monoclonal antihuman cyclin E antibody. Control whole-cell lysates (50 μg) from the HC11-V#1 (*Lane 1*) and the HC11-E#8 (*Lane 4*) cell lines are also shown. For additional details, see "Materials and Methods."

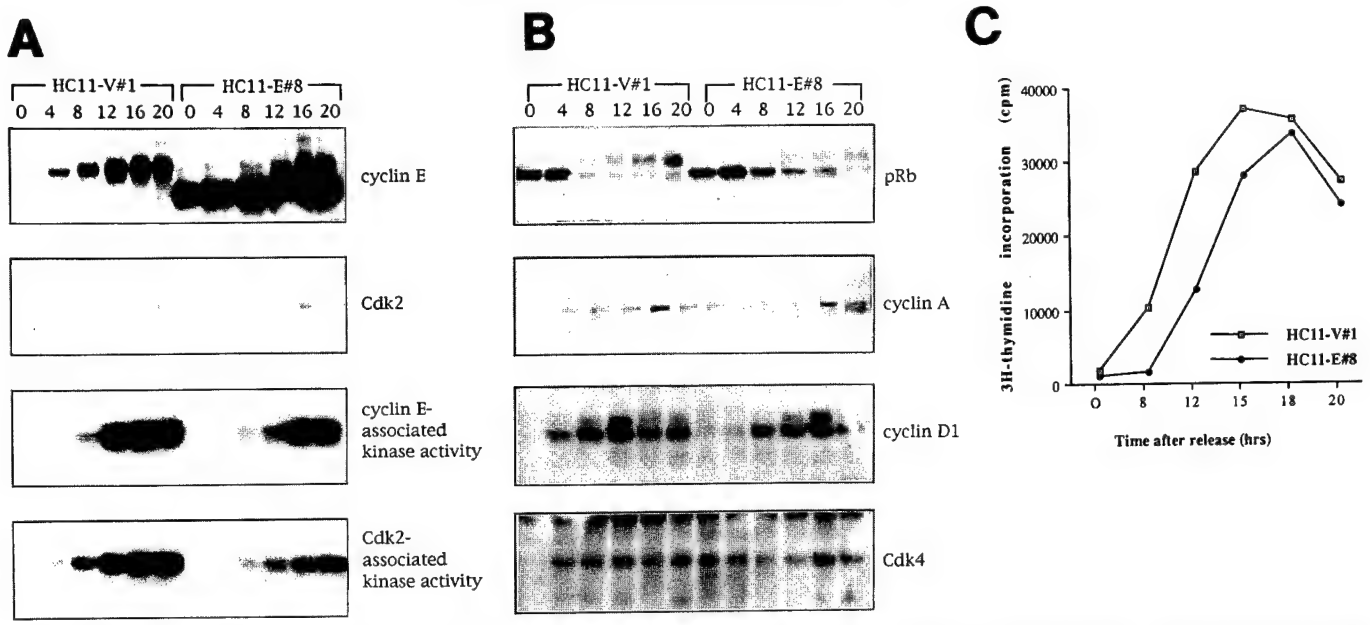

Fig. 3. Effects of stable overexpression of human cyclin E on events related to the G_0-to-S transition in HC11 cells. The HC11-V#1 vector control cell line and HC11-E#8 cyclin E-overexpressing cell lines were serum starved and then stimulated to reenter the cycle by refeeding with complete medium. *A*, whole-cell extracts were prepared from quiescent serum-starved cultures (*0*) and from cells harvested at the indicated times (in hours) after serum addition. Duplicate blots were reacted with anti-cyclin E and anti-Cdk2 polyclonal antibodies. The same antibodies were used to immunoprecipitate, respectively, cyclin E and Cdk2 immunocomplexes, and kinase activity was measured by using histone H1 as a substrate. *B*, cell extracts were prepared as in *A*. Duplicate blots were reacted with monoclonal anti-pRb and polyclonal anti-cyclin D1, anti-cyclin A, and anti-Cdk4 antibodies. *C*, DNA synthesis in triplicate cultures was monitored by measuring [^{3}H]thymidine incorporation into the acid-insoluble fraction after incubation for 1 h with [^{3}H]thymidine (1 μCi/ml) at the indicated times after serum addition (see "Materials and Methods" for details).

HC11 and vector control cells, the endogenous M_r 55,000 protein was induced maximally at about 12 h, whereas in the cyclin E-overexpressing cell lines, maximal induction of this protein was delayed to about 16 h, and its total level of expression was also somewhat suppressed. As expected, in the latter clones, the expression of the lower molecular weight cyclin E proteins encoded by the exogenous cyclin E cDNA was constitutive throughout this time course (Fig. 3*A*). Maximum induction of the cyclin D1 protein was also delayed by about 4 h in the cyclin E overexpressor clones (Fig. 3*B*). Assays for cyclin E-associated kinase activity at different times after serum stimulation indicated that in the control cells this activity was maximal at about 12 h, whereas in the cyclin E overexpressor clones it was maximal at about 16 h, thus paralleling the delay in induction of the endogenous M_r 55,000 cyclin E protein (Fig. 3*A*). A slight delay in the expression of cyclin A was also observed in the cyclin E overexpressor clones, but no significant differences were observed between the control and overexpressor clones in the expression of the Cdk2 and Cdk4 proteins (Fig. 3, *A* and *B*). However, the timing of Cdk2-associated kinase activity was delayed in the cyclin E-overexpressing cells (Fig. 3*A*), and a similar delay was observed also for the cyclin A-associated kinase activity (data not shown). In the control cell line, the pRb protein demonstrated a shift to the higher molecular weight hyperphosphorylated form (51) at about 8 h, whereas in the cyclin E overexpressor clones, this did not occur until about 12 h (Fig. 3*B*). This finding is consistent with the delayed induction of cyclin D1 and endogenous cyclin E and cyclin E-associated kinase activity (Fig. 3, *A* and *B*) in the overexpressor clones, since these events are implicated in the phosphorylation of the pRb protein (26, 27, 30). Thus, overexpression of exogenous cyclin E in the HC11 cells appears to have a feedback-inhibitory effect on several events involved in the G_0-to-S transition, at least in cells synchronized by serum starvation. Furthermore, constitutive expression of the exogenous cyclin E cDNA did not increase cyclin E-associated kinase activity or enhance the G_0-to-S transition.

The Cyclin E cDNA Used in This Study Reproduces the Effects Reported Previously When Overexpressed in Rodent Fibroblasts. These observations raised the possibility that the inhibitory effects on cell growth, together with the lack of increased cyclin E-associated kinase activity, might be due to a defect in the cDNA used in these studies, or that the human cyclin E protein was unable to bind to and activate the murine Cdk2. However, the latter possibility seemed unlikely since human and mouse cyclin E show a high degree of homology (52) and human cyclin E has been reported to activate rodent Cdk2 (10, 12, 17, 18).

To verify the integrity of the cDNA used in this study, we prepared a pair of primers for the regions flanking the *Hin*dIII site of PMV12pl, into which the cyclin E cDNA had been subcloned, amplified this region by PCR, and sequenced the inserted cDNA. No difference was detected between the sequence of the cDNA present in our PMV12-cycE construct and the sequence originally reported for the HU4 cyclin E cDNA (Ref. 23; data not shown).

As mentioned above, the same human cyclin E cDNA was overexpressed previously in Rat1 fibroblasts and showed very different effects (10, 17). To further examine the integrity of the cyclin E cDNA used in this study, we obtained Rat1 fibroblasts and transduced them with the same retrovirus constructs used to transduce the HC11 cells. Several independent pools of Rat1 cells transduced with either the PMV12pl or the PMV12-cycE constructs were selected by growth in hygromycin and then analyzed. Western blot analysis, using the polyclonal anti-cyclin E antibody, showed increased expression of a M_r 50,000 cyclin E protein in the latter derivatives, and this was associated with an increase in the amount of cyclin E-associated histone H1 kinase activity (Fig. 4). Using the monoclonal antibody specific for human cyclin E, we also detected the M_r 42,000 band seen in derivatives of HC11 cells (data not shown). When exponentially growing cells were examined by FACS analysis, unlike the HC11-cyclin E derivatives (Table 2), the cyclin E-overexpressing derivatives of Rat1 cells showed a decrease in the proportion of cells in G_1 (36%

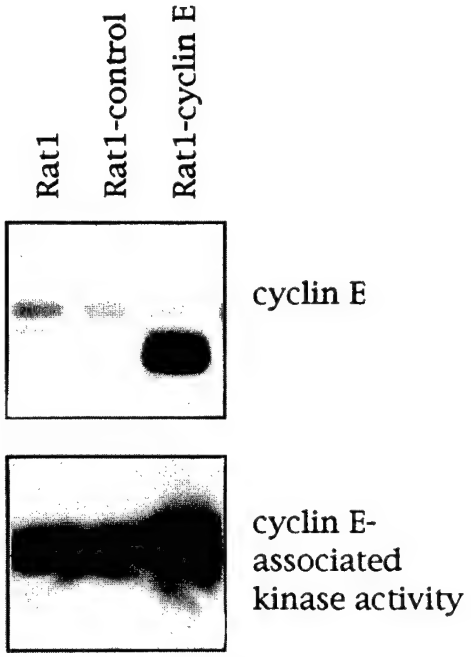

Fig. 4. Constitutive overexpression of human cyclin E in rat fibroblasts. Exponentially growing Rat1 fibroblasts were infected with the same virus supernatants used to infect the HC11 mouse mammary cells. *Top panel,* expression of cyclin E was determined by Western blot analysis in whole-cell extracts (50 μg) from exponentially growing cultures of the Rat1 parental cell line, Rat1 vector control cell line, and Rat1/cyclin E-infected cells. The higher band corresponds to the endogenous M_r 52,000–55,000 form of cyclin E, and the smaller band corresponds to the M_r 50,000 form of the exogenous cyclin E, which is highly overexpressed in the Rat1-cyclin E cells. In the *bottom panel,* the histone H1 kinase activity in anti-cyclin E immunoprecipitates was assayed as described in Fig. 2.

versus 58%) and an increase of cells in S phase (48% *versus* 30%), thus reproducing the results reported previously (10). Thus, we excluded the possibility that the above-described effects in HC11 cells were due to a defect in the cyclin E cDNA used to transduce these cells. Furthermore, we have found that overexpression of the same cyclin E cDNA in NIH3T3 cells led to increased cyclin E-associated histone H1 kinase activity (data not shown). Therefore, human cyclin E is capable of activating the murine Cdk2.

Cyclin E Overexpression Is Associated with Increased Expression of $p27^{Kip1}$ in Mammary Epithelial Cells. The slower G_0-to-S transition in serum-starved and restimulated HC11 cyclin E overexpressor cells could be explained, in part, by the delayed induction of endogenous cyclins D1 and E and the associated delay in phosphorylation of the Rb protein (Fig. 3). However, it was not apparent why, despite the fact that these cells constitutively express high levels of the exogenous cyclin E, they did not display an increase in cyclin E-associated kinase activity (Figs. 2 and 3). As mentioned above, this was not due to the inability of human cyclin E to bind to or activate murine Cdk2. The exogenous M_r 50,000 and M_r 42,000 cyclin E proteins were readily detected by immunoblotting in cell extracts from either exponentially dividing or synchronized HC11 overexpressor cells, following immunoprecipitation with either anti-cyclin E (Fig. 2) or anti-Cdk2 (Fig. 5*B*) antibodies.

Several CDIs have been identified recently (31, 32). Amongst them, the CDIs $p21^{Waf1}$ (also called Cip1, Pic1, Sd1, and Cap20) and $p27^{Kip1}$ (also called Ick and Pic2) preferentially bind to and inhibit the cyclin E-Cdk2 complex (53–56). Therefore, we examined the possibility that these proteins might play a role in inhibiting the activity of the exogenous cyclin E in the HC11 derivatives. Western blot analysis of exponentially dividing or synchronized cells indicated that the $p21^{Waf1}$ protein was barely detectable in both the control and cyclin E overexpressor HC11 cells (data not shown). When serum-starved and restimulated cells were examined for the expression of the $p27^{Kip1}$ protein, the vector control cells displayed a moderate level of this protein, which decreased at about 6 h after serum stimulation (Fig. 5*A*). The cyclin E overexpressor clones displayed a relatively high level of this protein at time 0, and this level rose to even higher levels at 4–16 h after serum stimulation and then declined somewhat. The results of a typical experiment are shown in Fig. 5*A* for the cell lines HC11-V#1 and HC11-E#8, but similar results were obtained with additional cyclin E overexpressor clones (HC11-E#2, HC11-E#12, and HC11-E#13; data not shown). Exponentially dividing cultures of the cyclin E-overexpressing clones also displayed increased expression of the $p27^{Kip1}$ protein when compared to the control clones (Fig. 6). Furthermore, with serial passage, the cyclin E overexpressor clones tended to display decreased levels of expression of the exogenous cyclin E, and this was associated with a decline in the level of the $p27^{Kip1}$ protein (data not shown).

We also obtained evidence that the $p27^{Kip1}$ protein was complexed to the exogenous cyclin E (Fig. 5*B*). Following restimulation of serum-starved cells, extracts were obtained at 0, 12, and 20 h, and immunoprecipitates were prepared with an antibody to Cdk2 or an antibody to $p27^{Kip1}$. These precipitates were then fractionated by SDS-PAGE and immunoblotted with the monoclonal antihuman cyclin E antibody. The results obtained (Fig. 5*B*) are consistent with the presence of a complex that contains the human cyclin E, murine Cdk2,

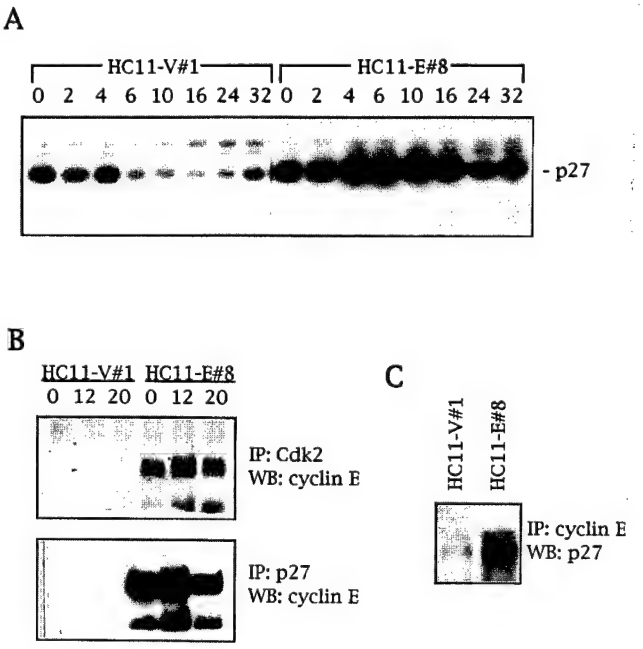

Fig. 5. Expression of $p27^{Kip1}$ in G_0-arrested and serum-stimulated vector control (HC11-V#1) and a cyclin E-overexpressing derivative (HC11-E#8) of the HC-11 cell line. *A,* cells were serum starved for 72 h, and total cell lysates were prepared from these quiescent cells (*0*) and at the indicated times after serum stimulation. Western blot analysis was performed as described in Fig. 1 using a polyclonal anti-$p27^{Kip1}$ antibody. *B,* association of the exogenous cyclin E with Cdk2 and $p27^{Kip1}$ in quiescent and serum-stimulated cells. Whole-cell extracts were prepared from the HC11-V#1 vector control and HC11-E#8 cyclin E-overexpressing cell lines after serum starvation (*0*) and at the indicated times (*12* and *20* h) after subsequent serum stimulation. Western blot analyses (*WB*) of either Cdk2 or $p27^{Kip1}$ immunoprecipitates were performed using the monoclonal antihuman cyclin E antibody. Immunoprecipitates (*IP*) were prepared by the incubation of total cell lysates (50 μg) with anti-Cdk2 or anti-$p27^{Kip1}$ polyclonal antibodies, respectively, as described in "Materials and Methods." *C,* an increased amount of p27 associates with cyclin E in the cyclin E-overexpressing cells. Whole-cell lysates (50 μg) from exponentially growing cultures of the HC11-V#1 vector control and HC11-E#8 cyclin E-overexpressing cell lines were immunoprecipitated (*IP*) using a polyclonal anti-cyclin E antibody. Immunoprecipitates were resolved by SDS-PAGE and immunoblotted with a polyclonal anti-$p27^{Kip1}$ antibody.

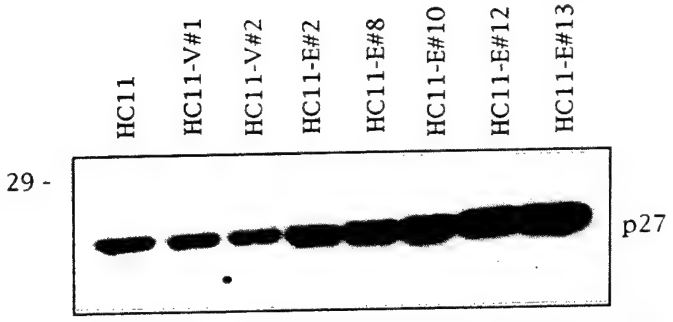

Fig. 6. Effects of stable overexpression of human cyclin E on the expression of $p27^{Kip1}$ in HC11 cells. Whole-cell extracts were prepared from exponentially proliferating cultures of the indicated cell lines. Fifty μg of protein were resolved by SDS-PAGE and transferred to an Immobilon membrane. The blot was probed with a polyclonal anti-$p27^{Kip1}$ antibody.

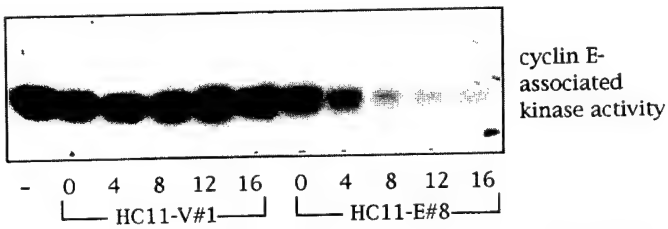

Fig. 7. A cyclin E-overexpressing derivative of the HC11 mouse mammary cell line expresses a heat-stable activity that can inhibit cyclin E-associated kinase activity. Whole-cell extracts were prepared from HC11-V#1 control and HC11-E#8 cyclin E-overexpressing cell lines after serum starvation (*0*) and at the indicated times after subsequent serum stimulation, as described in Fig. 5. An aliquot containing 50 μg of protein from each time point was boiled for 5 min, the precipitated proteins were cleared by centrifugation, and the remaining supernatant was mixed with a total cell extract (50 μg) prepared from vector control cells at 20 h after serum stimulation. Mixed lysates were assayed for cyclin E-associated kinase activity in the standard assay.

and $p27^{Kip1}$ protein in the cyclin E overexpressor but not the control HC11 clones. Evidence for this cyclin E-$p27^{Kip1}$ complex was also obtained in exponentially dividing HC11-E#8 cells (Fig. 5*C*). We found that the amount of the exogenous cyclin E protein in the immunoprecipitates obtained with the p27 antibody, from extracts of the cyclin E overexpressor cells, was approximately equivalent to the total amount of exogenous cyclin E present in the corresponding whole-cell lysates (data not shown). Thus, it appears that most, if not all, of the exogenous cyclin E is complexed with $p27^{Kip1}$. Therefore, the cyclin E overexpressor cells express much higher levels of $p27^{Kip1}$, and this inhibitory protein is also associated with the exogenous cyclin E. We could not accurately assess the amount of the endogenous cyclin E protein in these immunoprecipitates since it was obscured by the IgG band.

The $p27^{Kip1}$ protein is heat stable (54, 57). To verify the presence of increased amounts of a heat-stable inhibitory activity in the cyclin E-overexpressing HC11 cells, cell lysates were collected from HC11-V#1 control cells and HC11-E#8 overexpressor cells at different intervals during the G_0-to-S transition. The lysates were boiled for 5 min, and the precipitated proteins were removed by centrifugation. The remaining supernatant fractions were mixed with fixed amounts (50 μg) of cell lysate collected from control cells at a time point when cyclin E-associated kinase activity was very high (20 h). This mixture was then assayed for cyclin E-associated kinase activity (see "Materials and Methods"). Although not all of the $p27^{Kip1}$ protein was recovered after boiling the cell extracts (data not shown), heat-stable inhibitory activity was present in cell lysates collected at time zero from both control cells and cyclin E-overexpressing derivatives (Fig. 7). This inhibitory activity progressively decreased in control cells but increased at time points up to 16 h in the cyclin E-overexpressing cells, thus resembling the pattern of expression of the $p27^{Kip1}$ protein shown in Fig. 5*A*. Since $p27^{Kip1}$ is not the only heat-stable CDI, it is possible that other CDIs also contribute to this inhibitory activity. However, Western blot analyses failed to detect the recently identified $p57^{Kip2}$ CDI protein (58, 59) in the extract of the HC11 control or cyclin E overexpressor cells (data not shown).

Cyclin E and $p27^{Kip1}$ Expression Levels in Human Breast Cell Lines. Increased expression of cyclin E proteins has been reported in several types of human tumors, including breast cancer (34, 39, 40), and several human breast cancer cell lines (37). Therefore, it was of interest to examine whether deranged expression of the endogenous cyclin E in human breast cell lines was associated with increased expression of $p27^{Kip1}$. To test this idea, we examined, by immunoblotting, the levels of expression of cyclin E and $p27^{Kip1}$ proteins in several human cell lines originally derived from normal human mammary epithelial cells or breast carcinomas. As shown in Fig. 8, only the major M_r 52,000 form of cyclin E was expressed in the three nontumorigenic, immortalized mammary epithelial cell lines 184B5, 184A1 (42), and MCF-10F (60). The slight shift in this band is probably due to phosphorylation, as reported previously (25). On the other hand, all of the breast cancer cell lines examined (MCF-7, ZR-75–1, T47D, DU4475, and BT-549) showed increased expression of this M_r 52,000, as well as the presence of one or more lower molecular weight cyclin E-related proteins, including the M_r 50,000 and the M_r 42,000 forms seen in the cyclin E-overexpressing derivatives of the HC11 cell line (Fig. 8). These lower molecular weight, cyclin E-related proteins were also observed in the Hs578Bst and HBL-100 cell lines. The Hs578Bst cell line was derived from normal breast tissue peripheral to a carcinosarcoma, and because it is fibroblast like, it might be myoepithelial in origin (61). Although HBL-100 is an epithelial cell line originally derived from normal human mammary epithelium, it contains a tandemly integrated SV40 virus genome and is tumorigenic in nude mice (Refs. 46 and 62; ATCC catalog). It is of interest that the level of the $p27^{Kip1}$ protein was much higher in all of the breast cancer cell lines examined (Fig. 8, *Lanes 8–12*) than in the nontumorigenic, immortalized human mammary epithelial cell lines (Fig. 8, *Lanes 3–5*). Thus, aberrant expression of the endogenous cyclin E in these human cell lines is associated with increased expression of $p27^{Kip1}$.

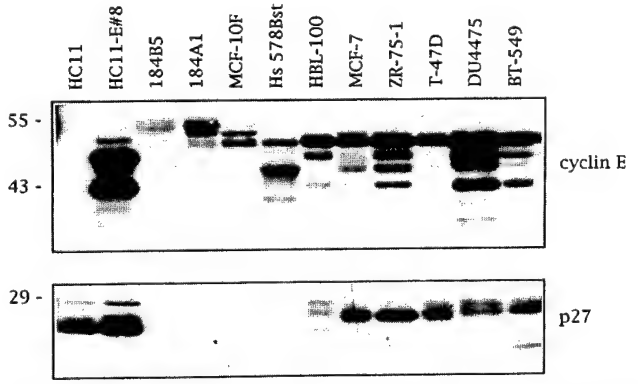

Fig. 8. Abnormal expression of cyclin E and $p27^{Kip1}$ proteins in human breast cancer cell lines. Western blot analysis for cyclin E (*top panel*) and $p27^{Kip1}$ (*bottom panel*) expression in normal immortalized human mammary epithelial cell lines (184B5, 184A1, MCF-10F, Hs578Bst, and HBL-100) and human breast cancer cell lines (MCF-7, ZR-75–1, T-47D, DU4475, and BT-549). A monoclonal antihuman cyclin E antibody was used, but a similar pattern was observed with a polyclonal anti-cyclin E antibody (data not shown). Faint bands corresponding to the M_r 50,000 and M_r 42,000 form of cyclin E proteins were visible in the normal cell lines after longer exposure. The murine $p27^{Kip1}$ protein (*Lanes 1* and *2*) had a slightly faster mobility than the human protein. Similar results were obtained with a different anti-$p27^{Kip1}$ antibody.

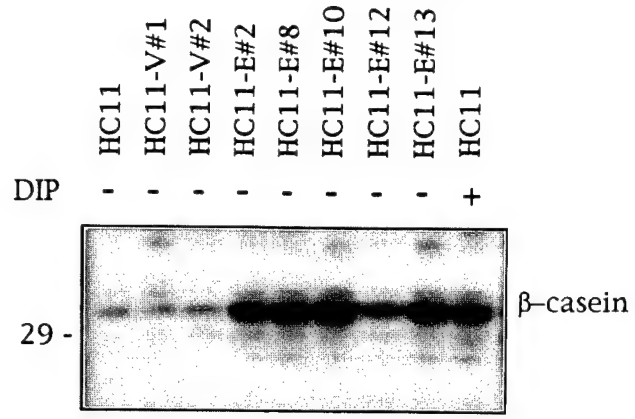

Fig. 9. Effects of stable overexpression of human cyclin E on the synthesis of β-casein in HC11 cells. The indicated cell lines were grown to confluency in the presence of EGF and then induced for 3 days with medium containing dexamethasone (*D*) and insulin (*I*) with or without prolactin (*P*). Cell extracts (75 μg) were resolved by SDS-PAGE and transferred to an Immobilon membrane; then the blot was probed with a rabbit antimouse casein antibody. For the HC11 parental cell line, β-casein expression is shown in the presence (+) and the absence (−) of prolactin. For all the other cell lines, β-casein expression is shown only in the absence of prolactin, but the results were the same when these cell lines were stimulated with complete DIP medium (data not shown).

Effects of Cyclin E Overexpression on the Production of β-Casein in HC11 Cells. HC11 cells are not transformed and can be induced to synthesize the β-casein milk protein when confluent cultures, previously exposed to EGF, are incubated in medium containing a mixture of dexamethasone, insulin, and the lactogenic hormone prolactin (DIP medium; Ref.41). They have provided, therefore, a useful *in vitro* model for studying the effects of different factors on mammary epithelial cell differentiation (63).

To determine whether cyclin E overexpression might affect the ability of these cells to synthesize β-casein, cyclin E-overexpressing derivatives and control cells were incubated with DIP (see "Materials and Methods") and the level of β-casein expression was determined by immunoblotting. The parental HC11 cells displayed a low level of spontaneous β-casein expression that was increased by the DIP induction (Fig. 9, compare *Lanes 1* and *9*). The cyclin E-overexpressing derivatives of HC11 cells showed a high level of spontaneous production of β-casein (Fig. 9), which was not further increased by hormone induction (data not shown). These findings suggest a possible relationship between cyclin E overexpression and differentiation in mouse mammary epithelial cells, although additional markers of differentiation remain to be examined.

DISCUSSION

To further address the role of cyclin E in mammary tumorigenesis, in the present study we developed derivatives of the normal mouse mammary epithelial cell line HC11, which stably overexpress a human cyclin E cDNA (HU4). We found that this causes the expression of two different forms of the cyclin E protein, a major band at about M_r 50,000 and a minor band at about M_r 42,000 (Fig. 1). The appearance of the smaller band was reported previously in derivatives of fibroblasts that overexpress the same human cyclin E cDNA, but this was seen only occasionally and was attributed to proteolytic degradation of the overexpressed M_r 50,000 band or to translation initiation at an internal methionine residue (10). With the use of a specific antihuman cyclin E antibody, we were able to demonstrate that the M_r 42,000 cyclin E was consistently expressed in parallel with the M_r 50,000 band in the derivatives of the HC11 cells. Furthermore, in some of these derivatives, we could detect the M_r 42,000 protein in clones that contained only low levels of the M_r 50,000 protein (Fig. 1), suggesting that it is not simply a minor degradation product. A second human cyclin E cDNA has been cloned recently that codes for a M_r 52,000 protein (17), and evidence has been obtained that the M_r 52,000 and M_r 50,000 proteins are encoded by alternatively spliced mRNAs (17). Overexpression of the M_r 52,000 cyclin E form in fibroblasts has been reported to cause the same effects as overexpression of the M_r 50,000 form (17). A M_r 43,000 splice variant of human cyclin E was identified previously in several human cell lines (64). We suggest that the M_r 42,000 band observed in the HC11/cyclin E-overexpressing derivatives (Fig. 1) is due to further splicing of the overexpressed cyclin E mRNA, since this mRNA contains both potential splice-donor and splice-acceptor sites (64). The existence of multiple cyclin E-related proteins that range in size from M_r 52,000 to about M_r 35,000 has been described previously in human cancer cells (Refs. 37 and 40; Fig. 8). The precise origin of these multiple forms of the cyclin E protein and their functional significance is not, however, apparent at the present time.

The present study demonstrates that overexpression of the same cyclin E cDNA can exert distinct biological effects in different cell types. Thus, whereas previous studies (10, 17) found that stable overexpression of the HU4 cyclin E cDNA in rat or human fibroblasts shortened G_1, decreased cell size, and enhanced growth, we found that stable overexpression of the same cDNA in the HC11 cells lengthened G_1, increased cell size, and inhibited growth (Tables 1 and 2; Fig. 3). Our findings are reproducible since they were seen with several clonal derivatives of HC11 cells that overexpress this cyclin E cDNA and were not seen in vector control clones (Tables 1 and 2). Furthermore, sequencing studies indicated that the present results are not due to mutations in this cDNA that might have occurred during construction of our PMV12-cycE plasmid. In addition, when we introduced the same construct into rat fibroblasts, we reproduced results obtained previously by Ohtsubo *et al.* (10, 17).

Further studies are in progress to determine whether the effects seen with the HC11 cells occur with other mammary epithelial cells. The divergent effects of this cyclin E cDNA in different cell types are not surprising since there are several other examples in which cell context influences the action of other genes involved in growth control. Thus, instead of enhancing growth, in certain cell lines the *ras* (65, 66), *fos* (67, 68), and *jun* (69) oncogenes can inhibit growth and induce differentiation; and the bcl-2 (70) and protein kinase Cβ (71) can have reciprocal effects in different cell types. Moreover, we reported recently that stable overexpression of a cyclin D1 cDNA in the HBL-100 human mammary epithelial cell line inhibits growth (46), whereas overexpression of the same cDNA in rat fibroblasts enhances their growth (9).

We believe that the most likely explanation for why the HC11-cyclin E derivatives have a prolonged G_1 phase and display growth inhibition is that stable overexpression in these cells of this human cyclin E cDNA induces, either directly or indirectly, the expression of the CDI protein $p27^{Kip1}$. The increased expression of this inhibitory protein was seen in the HC11-cyclin E cells when they were in continuous exponential growth and also in cultures synchronized by serum starvation and refeeding (Figs. 5 and 6). It was also a reproducible finding in several cyclin E-overexpressing clones and was not seen in the vector control clones. This interpretation is consistent with the fact that although these derivatives displayed a marked increase in the levels of the exogenous cyclin E proteins, extracts of these cells did not display an increase in cyclin E-associated kinase activity when compared to extracts from control cells (Fig. 2). Extracts of the overexpressor cells also displayed an increase in inhibitory activity in *in vitro* assays for cyclin E-associated kinase activity (Fig. 7). In addition, both the exogenous cyclin E protein and the $p27^{Kip1}$ protein

could be detected in cyclin E/Cdk2 immunoprecipitates obtained from the overexpressor cells (Fig. 5).

Although it was originally reported that $p27^{Kip1}$ is expressed at a constant level in murine fibroblasts (55), subsequent studies have shown that the level of $p27^{Kip1}$ increases in serum-starved cells and is down-regulated by growth factors (72, 73). Moreover, when quiescent T lymphocytes are stimulated to proliferate by the addition of interleukin 2, the level of this protein decreases (74, 75). This decrease in $p27^{Kip1}$ is prevented by rapamycin, a potent immunosuppressant that inhibits the G_1-to-S progression, presumably by preventing activation of the cyclin E/Cdk2 complex (75). Moreover, $p27^{Kip1}$ accumulates in Mv1Lu mink epithelial cells arrested in G_0-G_1 due to cell-cell contact or treatment with transforming growth factor β (57, 76, 77), and overexpression of exogenous $p27^{Kip1}$ in mink Mv1Lu or human Saos-2 osteosarcoma cells arrests cells in G_1 (54, 55). There is also evidence that dislocation of $p27^{Kip1}$ from the cyclin E/Cdk2 complex is essential for the activation of kinase activity and that this is also prevented by transforming growth factor β treatment (57, 76). All of these findings are consistent with our hypothesis that the increased levels of $p27^{Kip1}$ in the HC11/cyclin E-overexpressing cells may explain several aspects of their phenotype.

We further hypothesize that the increased expression of $p27^{Kip1}$ in the HC11/cyclin E-overexpressing cells is a manifestation of a positive feedback loop between cyclin E and $p27^{Kip1}$, which is present in HC11 cells but may be absent or have a different set point in fibroblasts. Consistent with this hypothesis is the fact that rat fibroblasts that stably overexpress the same human cyclin E cDNA do not display an increase in the $p27^{Kip1}$ protein (data not shown). The increase in $p27^{Kip1}$ in the HC11-cyclin E overexpressor cells might protect these cells against potentially toxic effects of cyclin E overexpression, thus enhancing their viability although they grow more slowly. It is of interest that with prolonged serial passages, the HC11-cyclin E#10 and E#12 clones displayed a loss of expression of the exogenous cyclin E, and this was associated with decreased expression of $p27^{Kip1}$ (data not shown). Further studies are required to definitively establish that overexpression of cyclin E directly induces $p27^{Kip1}$ expression in HC11 cells and that this association is not simply due to cell selection.

We should also emphasize, however, that the unusual effects seen in the present study might be peculiar to ectopic expression of specific cyclin E proteins and may not play a role in normal cell physiology. Thus, the parental HC11 cells mainly express a M_r 55,000 form of cyclin E and the M_r 50,000 form is expressed at a very low level (Fig. 1). Moreover, only the M_r 55,000 band undergoes specific cell cycle regulation, both in terms of its level of expression and phosphorylation (Fig. 3). On the other hand, the cyclin E-overexpressing derivatives express high levels of the M_r 50,000 and M_r 42,000 human cyclin E proteins. In addition, the ectopic cyclin E proteins are expressed constitutively throughout the cell cycle, in contrast to the endogenous cyclin E proteins (Fig. 3). This temporal difference might also perturb normal cell cycle regulatory events and the expression of $p27^{Kip1}$.

The present model system may, however, be instructive for revealing regulatory loops that apply in certain circumstances. Thus, as discussed above, certain tumor cells and some normal cell lines often display dysregulation in the expression of their endogenous cyclin E gene, and these cells also accumulate lower molecular weight cyclin E proteins. Some of these proteins might be defective in binding to and activating Cdk2, like the M_r 42,000 protein (64), but they could also play a role in feedback regulation of cell cycle progression. It is of interest that the HC11 derivatives that express the exogenous cyclin E cDNA display not only increased expression of the endogenous $p27^{Kip1}$ but also decreased expression of their endogenous cyclin E protein (Figs. 1 and 3). Additional studies are required to determine if this represents an independent negative feedback loop in the control of cyclin E expression or is a secondary effect due to changes in cell cycle control.

Finally, the findings in the present study may be relevant to human breast cancer since we have found that a series of tumorigenic human breast cancer cell lines express a high level of $p27^{Kip1}$ and that the same cell lines display increased expression of lower molecular weight forms of cyclin E (Fig. 8). The high level of expression of $p27^{Kip1}$ is surprising since this protein is thought to act as a tumor suppressor (2). Thus, it is possible that our hypothesis of a positive feedback loop between cyclin E and $p27^{Kip1}$ applies to these cell lines. Studies are in progress to further evaluate the significance of this association in human breast cancer.

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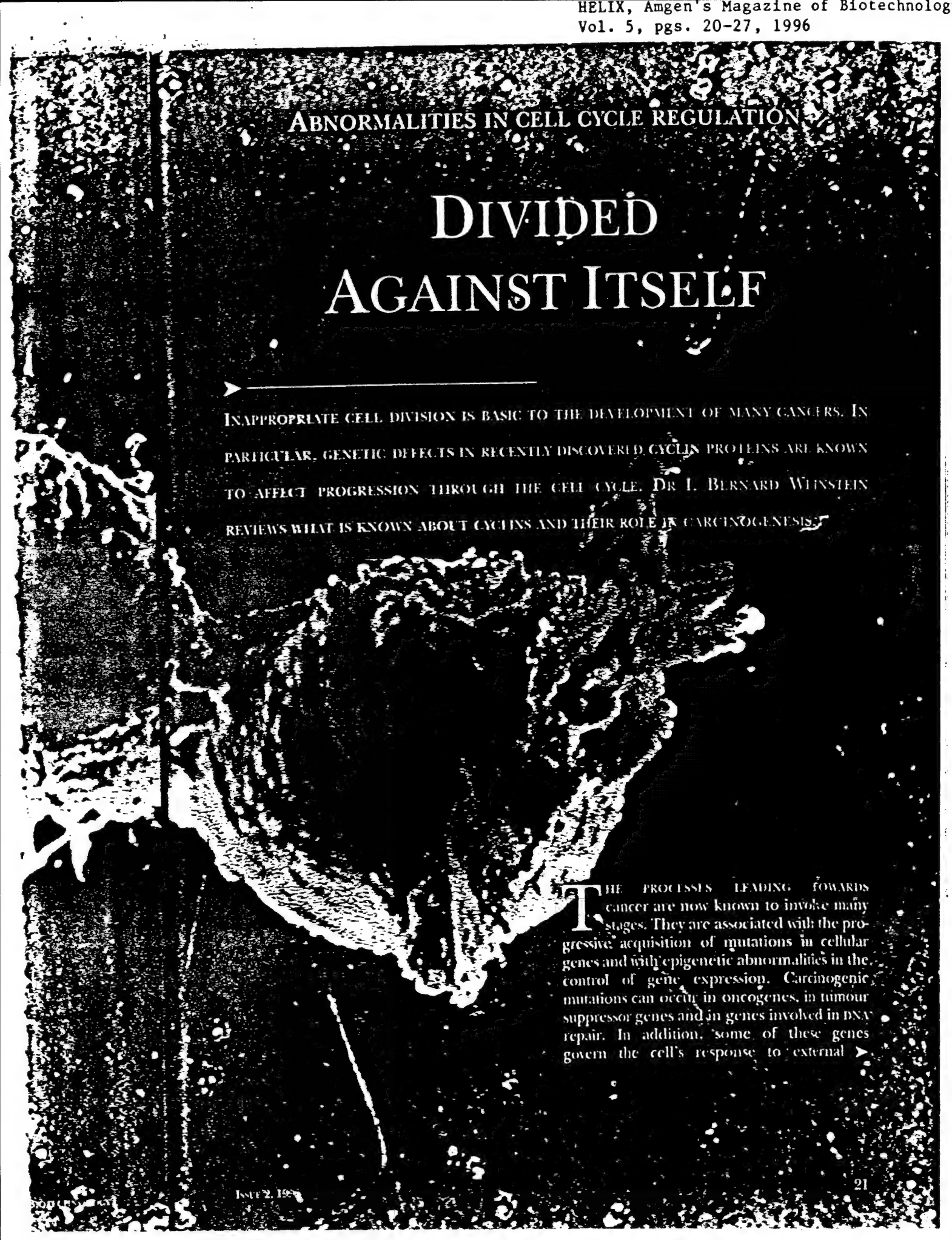
ABNORMALITIES IN CELL CYCLE REGULATION

DIVIDED AGAINST ITSELF

INAPPROPRIATE CELL DIVISION IS BASIC TO THE DEVELOPMENT OF MANY CANCERS. IN PARTICULAR, GENETIC DEFECTS IN RECENTLY DISCOVERED CYCLIN PROTEINS ARE KNOWN TO AFFECT PROGRESSION THROUGH THE CELL CYCLE. DR I. BERNARD WEINSTEIN REVIEWS WHAT IS KNOWN ABOUT CYCLINS AND THEIR ROLE IN CARCINOGENESIS.



THE PROCESSES LEADING TOWARDS cancer are now known to involve many stages. They are associated with the progressive acquisition of mutations in cellular genes and with epigenetic abnormalities in the control of gene expression. Carcinogenic mutations can occur in oncogenes, in tumour suppressor genes and in genes involved in DNA repair. In addition, some of these genes govern the cell's response to external ➤

False-colour scanning electron micrograph of two human cells in the final stage (telophase) of cell division. Cellular growth control and proliferation can be perturbed by aberrations in the proto-oncogenes and tumour suppressor genes which control the cell cycle.

CYCLINS GOVERN THE ORDERLY PROGRESSION OF MAMMALIAN CELLS THROUGH THE CELL CYCLE.

Onion root-tip cells in different phases of cell division. Cyclins were first discovered in studies of lower organisms.

growth factors, since they encode for growth factors themselves, for growth factor receptors, for proteins involved in signal transduction pathways in the cytoplasm, or for nuclear transcription factors. To an extent, they determine whether cells will be in the resting, non-dividing G0 state or whether they will enter into the active G1 phase of the cell cycle to undergo replication and proliferation.

It is becoming increasingly apparent that another group of proto-oncogenes and tumour suppressor genes is involved in controlling the cell cycle once it is actually underway. Aberrations in these genes, which are particularly active during the late G1 and early S phases of the cycle, can also perturb cellular growth control and proliferation. Moreover, these aberrations might contribute to genomic instability, thereby enhancing tumour progression and heterogeneity.

The proteins involved in regulating progression through the cell cycle include cyclins, cyclin-dependent kinases (CDKs), CDK inhibitors (CDIs) and other cell-cycle associated proteins such as Rb (the product of the retinoblastoma gene) and p53 (the product of the *p53* tumour suppressor gene). This article briefly reviews what is known about the role of these proteins in promoting and controlling the cell cycle and examines possible clinical implications.

CYCLINS, KINASES AND INHIBITORS

CYCLINS WERE ORIGINALLY DISCOVERED IN studies of cell division in lower organisms. There is now abundant evidence that the orderly progression of mammalian cells through the G1, S and G2/M stages of the cell cycle is also governed by specific cyclins. Although cyclins do not themselves act as enzymes, they exert their influence by binding to and activating CDKs. The name cyclin derives from the fact that the levels of these proteins oscillate throughout the cell cycle and, as a result, they cyclically regulate the activity of CDKs, which tend to be present in excess.

The cell cycle requires accurate timing and coordination. In mammalian cells, fine control is achieved by checkpoints at the G1/S (before DNA is duplicated) and the G2/M transitions (before mitosis starts). These checkpoints delay progress through the cell cycle to provide an opportunity for the repair of defects, including DNA damage, caused by potentially cytotoxic events. CDIs and other proteins play a role at these checkpoints. For example, both Rb and p53 are important at the G1/S checkpoint. Rb acts by binding the nuclear transcription factor E2F, while p53 induces the synthesis of a CDI, $p21^{WAF1}$, and other proteins. Inactivating mutations in the *Rb* and *p53* genes occur frequently in a number of human cancers.

Very little is known about the G2/M checkpoint in mammalian cells but it is

Cyclin D1 is the cyclin most frequently implicated in the development of human tumours.

Figure 1. A schematic diagram of the mammalian cell cycle indicating the G0 phase of non-dividing cells, the G1 phase where cells enter the cell cycle and prepare for DNA synthesis (S phase), and the G2 and M phases in which cells prepare for and undergo mitosis. Also shown are the cyclins and cyclin-dependent protein kinases (CDKs) that regulate the cell cycle at specific stages, and the G1/S checkpoint at which the Rb and p53 tumour suppressor genes can inhibit cell cycle progression.

conceivable that defects arising at this stage may contribute to the chromosomal anomalies seen in malignant tumours.

Several cyclin genes have now been identified. They are classified into three major groups: G1, A and B cyclins. The G1 cyclins (cyclins D1, D2, D3 and E) are maximally expressed during the G1 phase and regulate progression of the cell cycle from the mid-G1 phase into the S phase (*see figure 1*). Cyclin A is highly expressed in the early S phase and enhances progression through that phase. It also acts during the G2/M transition. Two B-type cyclins (cyclins B1 and B2) are important for the entry and exit of cells from mitosis (M phase). The specific roles of four additional cyclins, cyclins C, F, G and H, are still being explored and the functional significance of the multiple D and B cyclins is not known.

Cyclins function by binding to and stimulating the activity of their catalytic partners, the cyclin-dependent kinases (*figure 2*). CDK activation additionally requires phosphorylation at a conserved threonine residue (Thr161 in CDK1 and Thr172 in CDK4 and CDK6). In contrast, phosphorylation at other sites (e.g. Thr14 or Tyr15) can inhibit cyclin-CDK activity.

To date, at least eight mammalian CDKs, designated CDK1 to CDK8, have been identified. CDK1 (also called CDC2) can associate with cyclin B or cyclin A. The resulting complexes appear to be involved in regulation of the G2/M transition. CDK2, in association with cyclins E and A, is involved in regulating G1/S transition and S phase progression, respectively. CDK4 and CDK5 are the major catalytic partners for cyclins D1, D2 and D3. The resulting cyclin-CDK complexes can phosphorylate Rb. CDK5 also forms complexes with the D cyclins but the functions of these complexes are not known.

The protein $p21^{WAF1}$ (also called CIP1) is one of several CDK inhibitors which have been recently discovered. $p21^{WAF1}$ synthesis is induced by the accumulation of the p53 protein in response to DNA damage. It brings about cell-cycle arrest by binding to and inhibiting the activation of a number of cyclin-CDK complexes (*see figure 2*), including the cyclin D1-CDK4, cyclin D1-CDK6, cyclin E-CDK2 and cyclin A-CDK2 complexes. Similarly, another inhibitor, the protein $p27^{KIP1}$ arrests the cell cycle at the G1/S transition by binding to and inactivating the cyclin D1-CDK4, cyclin D1-CDK6 and cyclin E-CDK2 complexes. This process takes place when cells experience growth inhibition due to cell-to-cell contact or in response to treatment with transforming growth factor-β (TGF-β). In human keratinocytes, it appears that the effect of TGF-β is mediated ➤

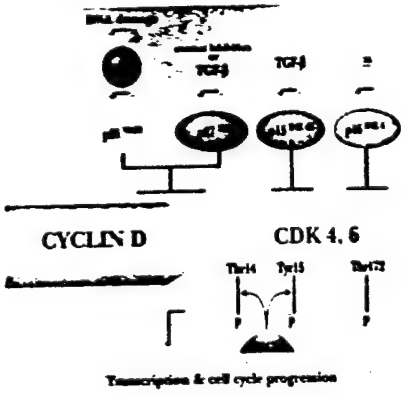


Figure 2. Multiple mechanisms regulate cyclins and CDKs. The binding of a cyclin to a CDK can activate its kinase activity. Kinase activity can be inhibited by phosphorylation (by specific protein kinases) of the CDK on a tyrosine residue at position 15 (Tyr15) or on a threonine residue at position 14 (Thr14). Removal of these phosphate residues by CDC25 phosphatases relieves this inhibition. In contrast, phosphorylation of a CDK at a threonine residue at position 160 or 161 or 172, depending on the particular CDK) by cyclin H-CDK7 (also called CAK) enhances the kinase activity. A group of inhibitory proteins (CDIs) designated $p16^{INK4}$, $p15^{INK4B}$, $p21^{WAF1}$ and $p27^{KIP1}$ (and other related proteins) bind to specific cyclin-CDK complexes and inhibit their kinase activity. Various external factors acting through the CDIs can bring about cell cycle arrest.

INCREASED EXPRESSION OF CYCLIN D1 CAN BE DETECTED AT AN EARLY STAGE IN COLON CARCINOGENESIS.

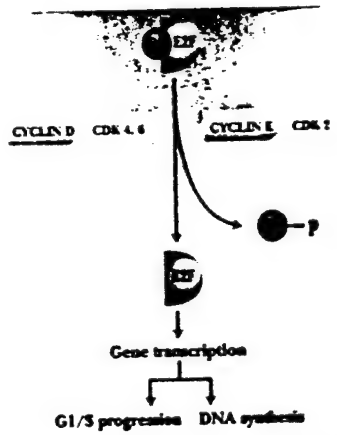


Figure 3. In the early G1 phase the transcription factor E2F is inactive because it is bound to the Rb tumour suppressor protein. Phosphorylation of Rb by activated cyclin D-CDK4 (or CDK6) and cyclin E-CDK2 leads to the release of E2F, which can then activate transcription of the genes involved in late G1 to S progression and the genes involved in DNA synthesis during the S phase of the cell cycle.

by a protein designated p15 (also called INK4B or MTS2). Another inhibitor $p16^{INK4}$ (also called MTS1) is known to function by binding to and inhibiting the activities of CDK4 and CDK6. A number of other CDIs have also been identified recently, including p18 and p19, which are related to p15 and $p16^{INK4}$, and p57, which is related to $p27^{KIP1}$.

Cyclins, as well as binding to CDKs, can also bind to other proteins which influence the cell cycle. For example, cyclin D1, the cyclin most frequently implicated in the development of human tumours, can form complexes with the DNA replication factor proliferating-cell nuclear antigen (PCNA) and with the retinoblastoma protein (Rb). Several studies indicate that cyclin D1 is involved in inactivating Rb and, thereby, abrogating its inhibitory effect on G1/S progression. When Rb is phosphorylated it no longer binds the transcription factor E2F. E2F can then act to turn on the expression of genes required for further cell cycle progression (*see figure 3*). Furthermore, cyclin D1 gene expression is positively regulated by the Rb protein. The result is that Rb and cyclin D1 form an autoregulatory feedback loop.

ABNORMALITIES IN CANCER

ABNORMALITIES IN CYCLINS AND CYCLIN-RELATED genes have now been implicated in several types of human tumours (*see table*). One of the earliest examples was the finding that the cyclin A gene was the site for integration of the hepatitis B virus (HBV) in a human hepatocellular carcinoma. This led to the synthesis of a stable HBV-cyclin A fusion protein. This abnormality appears, however, to be a rare event in liver cancer.

Abnormalities in the cyclin D1 gene, in contrast, have been associated with numerous human cancers. The cyclin D1 gene is amplified and overexpressed, at both the mRNA and protein levels, in a significant fraction of primary human breast carcinomas, oesophageal carcinomas, squamous carcinomas of the head and neck, non-small cell lung carcinomas, hepatocellular carcinomas and bladder carcinomas. The cyclin D1 gene, also termed *prad-1* or *bcl-1*, is located at chromosome 11q13. Chromosomal rearrangements at this locus found in parathyroid tumours and centrocytic B-cell lymphomas cause increased and constitutive expression of this gene.

Several types of mechanistic studies specifically implicate the cyclin D1 gene in tumorigenesis. Gene transfer studies, for example, have shown that stable overexpression of cyclin D1 in rodent fibroblasts enhances their

False-colour transmission electron micrograph of hepatitis B virus particles. The cyclin A gene was found to be the site of integration of the hepatitis B virus in a human hepatocellular carcinoma.

tumorigenicity in nude mice, and transfection studies indicate that cyclin D1 cooperates with a defective adenovirus E1A or an activated *ras* oncogene in the transformation of rodent cell lines. Additional evidence for the critical role of cyclin D1 overexpression comes from studies in our laboratory where we have demonstrated that expression of an antisense cyclin D1 sequence in a human oesophageal cancer cell line (one in which the endogenous cyclin D1 gene is amplified and overexpressed) causes reversion of the transformed phenotype and loss of tumorigenicity.

Both amplification and increased expression of cyclin D1 are found in about fifteen per cent of primary human breast cancers. Increased expression of cyclin D1 alone, in the absence of gene amplification, occurs in an additional 45 per cent of human breast carcinomas and in about forty per cent of human colon carcinomas. In colon tumours, this appears to be an early event since it occurs in around forty per cent of adenomas of the colon and in about the same proportion of Dukes stages A, B, C, and D carcinomas. The mechanism responsible for increased expression in the absence of gene amplification is not known. It is of interest that activated *ras* or *myc* oncogenes can induce increased expression of cyclin D1 in cell culture systems. Accumulation of the p53 tumour suppressor protein in response to DNA damage can also induce increased expression. On the other hand, p53 accumulation and the subsequent induction of $p21^{WAF1}$ usually inhibit cell cycle progression.

Another gene, the cyclin E gene, is often dysregulated and overexpressed in a variety of human carcinomas. With rare exceptions, it is usually not amplified. Human tumours often express a series of lower molecular weight forms of the cyclin E protein, which are due, at least in part, to variations in mRNA splicing.

In addition to abnormalities in cyclin genes, well-documented examples of abnormalities in a cyclin-dependent kinase gene and in a gene which modulates the phosphorylation state of CDK proteins have been found in human cancers. Between ten and thirty per cent of gliomas and sarcomas, for example, display amplification and increased expression of the CDK4 gene. Since, in these tumours, the amplified CDK4 gene is part of a much larger amplicon located at chromosome 12q13, it is possible that other amplified genes in this region (for example *MDM2* and *GLI*) also contribute to tumorigenesis. A recent report described the occurrence of a CDK4 mutation in human melanomas which makes the protein resistant to inhibition by the $p16^{INK4}$ inhibitor. Interestingly, mutant CDK4 acts like a tumour-specific antigen and is recognized by T lymphocytes.

Genes for proteins which promote CDK activity through a process of dephosphorylation have also been implicated in cancer. During the cell cycle, a series of CDC25-phosphatases remove phosphate residues from ➤

Table 1 CYCLINS AND CYCLIN-RELATED ABNORMALITIES IN HUMAN CANCER

Gene	Type Of Abnormality	Types Of Cancers
Cyclin D1	Chromosomal translocations cause constitutive activation	Parathyroid tumours, B-cell lymphomas
	Gene amplification and increased expression	Breast, oesophagus, squamous carcinomas of the head and neck, non-small-cell lung, liver, bladder
	Increased expression without amplification	Breast, colon
Cyclin E	Increased expression and aberrant low molecular weight forms[1]	Breast, colon, prostate
Cyclin A	Site of integration of hepatitis B virus, yielding a fusion protein[2]	Liver
CDK4	Amplification and increased expression	Gliomas and sarcomas
	$p16^{INK4}$-insensitive mutant	Melanoma
CDC25B	Increased expression	Breast
$p16^{INK4}$	Loss of expression due to deletions, point mutations or methylation	Pancreas, oesophagus, gliomas, leukaemias

Note that inactivating mutations in the *Rb* and *p53* genes also occur in a wide variety of human tumours.
1 Amplification of cyclin E is only seen rarely in colon and breast cancer cell lines. Dysregulation in the expression of cyclins E, D1 and A, however, occurs in a variety of human cancer cell lines.
2 Only a single case of this event has been reported.

➤ *Between ten and thirty per cent of gliomas and sarcomas display amplification and increased expression of the CDK4 gene.* ◄

CYCLINS AND CYCLIN-RELATED KINASES MAY PROVIDE NOVEL TARGETS FOR CANCER PREVENTION AND THERAPY.

the tyrosine-15 and threonine-14 sites where they normally inhibit the kinase activity of CDKs (*see figure 2*). Mammalian cells have three CDC25 genes, designated A, B, and C. Increased expression of CDC25B has been found in several tumour cell lines and in about thirty per cent of primary human breast tumours. A high level of expression of CDC25B correlates with a poor prognosis. Further, transfection studies indicate that CDC25A and B, but not C, cooperate with either an activated *ras* oncogene, or the loss of the *Rb* gene, to transform murine fibroblasts.

It would seem likely that a loss of expression of the proteins which inhibit CDK activity, the CDIs, would also contribute to tumorigenesis. Indeed, the gene for the $p16^{INK4}$ protein, which binds to and inhibits CDK4 and CDK6, is located on chromosome 9p21, a chromosomal region known to be deleted in several types of human cancer. In addition, the $p16^{INK4}$ gene is frequently deleted in human cancer cell lines. There is some controversy, however, about whether or not this specific gene deletion is common in primary human tumours. Nevertheless, recent studies have shown a loss of $p16^{INK4}$ expression in familial melanomas, gliomas, leukaemias, and pancreatic and several other types of cancer. Although mutations have so far not been seen in the genes that encode the CDIs $p21^{WAF1}$ and $p27^{KIP1}$, mutations in the p53 tumour-suppressor gene can impair the induction of $p21^{WAF1}$ in response to DNA damage.

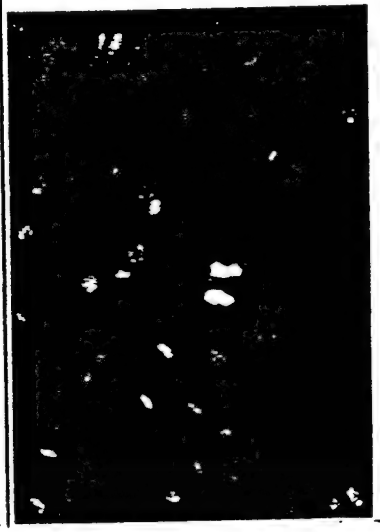
CLINICAL IMPLICATIONS

THE DISCOVERY THAT ABNORMALITIES IN CYCLIN and cyclin-related genes occur in human cancers may open the way for new approaches to cancer diagnosis, prognosis and therapy. Increased expression of cyclin D1, in particular, may be important as it occurs frequently in several common types of tumour.

Negative feedback control pathways may provide mechanisms for buffering the effects of the excess expression of genes regulating the cell cycle. The interactions between cyclin D1 and the Rb or p53 proteins are examples of these feedback systems. The Rb and p53 proteins themselves inhibit G1/S progression but they also induce increased expression of cyclin D1, which in turn enhances G1/S progression. An increased Rb level also has an inhibitory effect on the expression of the CDK inhibitor protein $p16^{INK4}$.

Studies on oesophageal carcinomas have shed more light on these feedback pathways. Our laboratory found that the subset of tumours displaying amplification and increased expression of cyclin D1 have normal expression of the *Rb* gene, whereas, conversely, the subset of tumours not expressing the Rb protein (presumably due to deletion mutations) do not show amplification and increased expression of cyclin D1. It would appear that during the clonal evolution of tumour cells, the inhibitory effect of the *Rb* gene on cell cycle progression can be abrogated either by increased expression of cyclin D1, which would increase Rb protein phosphorylation and inactivate its inhibitory function, or by actual loss of the Rb protein itself.

A single regulatory pathway can, therefore, be perturbed by mutations in different genes which influence the pathway. An alternative mechanism during oesophageal carcinomas could be inactivation of the CDIs that act on cyclin D1-CDK4 and cyclin D1-CDK6 complexes. It appears that amplification and overexpression of CDK4 is often reciprocally associated with deletions of the CDI protein $p16^{INK4}$. These complex interrelationships may explain why different tumours of the same histological type can have a different spectrum of gene mutations. In the design and use of new gene-specific anticancer agents, therefore, it may be



In these neuroblastoma cancer cells the cytoplasm stains red, the nuclei blue and normal epithelial cells green. CDK4 gene expression is increased in some neurological cancers.

Immunofluorescent light micrograph of a squamous cell carcinoma. Nuclei stain blue, the cytoplasm red and the cell membrane green. The cyclin D1 gene is amplified and overexpressed in a significant fraction of squamous carcinomas of the head and neck.

necessary to identify the specific mutation involved in a particular tumour or to design agents that are pathway-specific rather than gene-specific.

Cyclins and cyclin-related kinases may also provide novel targets for cancer prevention and therapy. Human tumours often display amplification and increased expression of several genes, including cellular oncogenes and genes conferring drug resistance. Gene amplification is, therefore, an important cause of tumour progression and heterogeneity. Studies in our laboratory have recently demonstrated that increased cyclin D1 expression can enhance the process of gene amplification, suggesting that cyclin D1 might play a critical role in the genomic instability often associated with tumour progression. Inhibitors of cyclin D1 or cyclin D1-CDK kinase activity might, therefore, prevent genomic instability and tumour progression. These findings should encourage the development of specific drugs that inhibit the activity of cyclin D1, CDK4 and related factors as a new approach for treating cancer.

Finally, cyclins and cyclin-related kinases may have a role in cancer prognosis or in predicting the responses of specific tumours to therapy. In particular, increased expression of cyclins D1 and E, CDK4 or CDC25B could serve as prognostic markers in clinical studies. The finding that increased expression of cyclin D1 can be detected at an early stage in colon carcinogenesis indicates that it may be a useful marker in cancer detection and chemoprevention studies. In addition, studies with an antisense cyclin-D1 construct in our laboratory have demonstrated that increased expression of cyclin D1 is necessary for maintaining a malignant phenotype in some cancer cells.

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Relevance of Cyclin D1 and Other Molecular Markers to Cancer Chemoprevention

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Abstract Until recently studies on mutations in cellular genes implicated in multistage carcinogenesis have concentrated mainly on dominant acting mutations in cellular proto-oncogenes, genes that normally mediate agonist-induced signal transduction pathways, and recessive mutations in cellular tumor suppressor genes, whose normal products appear to inhibit cell growth and/or control differentiation and cell-cell interactions. It seems likely, however, that a third category of cellular genes, the cyclins and cyclin-related genes, may also be critical targets during multistage carcinogenesis because of the central role that they play in controlling cell cycle progression. These proteins could, therefore, provide biomarkers for identifying individuals at high risk of developing cancer and also serve as novel targets for chemopreventive agents. This paper reviews evidence that the gene cyclin D1 is amplified and/or overexpressed in a major fraction of human tumors, and that this can occur relatively early in the carcinogenic process. Mechanistic studies indicates that this overexpression plays a critical role in tumor progression as well as the maintenance of the tumorigenic phenotype. Thus, increased cyclin D1 expression can enhance gene amplification and cell transformation and antisense to cyclin D1 can revert malignant cells. The latter findings provide direct evidence that cyclin D and related proteins might be useful markers and also targets for cancer chemoprevention. J. Cell. Biochem. 25S:23–28.

Key words: cancer; cell cycle; chemoprevention cyclins; markers; prevention

AN OVERVIEW OF CANCER RISK FACTORS

This conference emphasizes the important topic of the identification of high risk individuals or subpopulations that might be targeted for cancer chemoprevention trials. We now know that multiple factors, both endogenous as well as exogenous, can act, often in combination, to influence the multistage process of carcinogenesis [for review see references 1–3]. Therefore, identification of such individuals is a challenging and complex task. Table I summarizes some of the major factors, known and hypothetical, that influence cancer risks. The category "Inheritance of Predisposing Genes" includes the familial cancer syndromes, for example, adenomatous polyposis coli, hereditary non-polyposis coli, and hereditary breast cancer (BRCA 1 and 2), which involve the inheritance of a single dominant acting gene. This category, although it represents only about 10% of all cancers, provides a valuable model for piloting chemoprevention studies because of the high penetrance of the inherited gene and the fact that molecular diagnostic tools are becoming available to identify with great certainty the individuals at risk. Of greater numerical importance, however, with respect to cancer risks in the general population is the inheritance of genes that influence cancer susceptibility via a multifactor mechanism, by influencing the response of the host to endogenous or exogenous carcinogenic factors. There is, for example, increasing evidence that specific polymorphic forms of drug metabolizing enzymes (both phase 1 and phase 2) can influence the susceptibility of individuals to the carcinogenic effects of cigarette smoke [for review see 2,3]. It also seems likely, that specific polymorphic forms of enzymes that play a role in DNA repair, or polymorphisms in proteins that influence the responses of cells to

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growth factors (i.e., receptors, protein kinases, and transcription factors), also influence cancer susceptibility, but this remains to be established. Individuals at high risk also include, of course, those with a history of heavy exposure to various external carcinogens, including cigarette smoke, specific occupational carcinogens, and radiation, and individuals with certain reproductive, lifestyle, or nutritional histories. Microbial agents constitute a category of cancer risk factors that is gaining increasing importance, because of the evidence for a causative role of hepatitis B and C in liver cancer, EBV virus in nasopharyngeal cancer and specific lymphomas, human papilloma virus in cervical cancer, and *Helicobacter pylori* in gastric cancer [2,3]. It also seems likely that specific bacteria in the intestinal flora play a role in colon cancer, through the production of diacylglycerol [1,2] and possibly specific mutagens, but this remains to be established. I believe that further efforts should be directed towards identifying the possible roles of specific microbial agents as cofactors in the causation of breast, prostate, and other prevalent forms of human cancer.

I want to also emphasize that because the majority of human cancers result from interactions between one or more of the above factors, the identification of individuals at high risk will often require scoring for two or more risk factors, as is now routinely the case in the field of cardiovascular disease. Thus, the risk of lung cancer in cigarette smokers may be especially high in individuals with specific polymorphic forms of drug-metabolizing enzymes, the risk of liver cancer may be especially high in individuals with chronic hepatitis B virus infection who have also had exposure to aflatoxin or other chemical carcinogens, and the risk of gastric cancer may be especially high in individuals with chronic *Helicobacter pylori* gastritis who have also had exposure to nitrosamines and suffer vitamin deficiencies [1–3]. Molecular epidemiology approaches [1–3] that employ epidemiologic methods in combination with markers for each of the suspected factors will, therefore, be required to identify with precision the individuals who are truly at high risk.

CATEGORIES OF GENES THAT ARE TARGETED DURING CARCINOGENESIS

Another approach to identifying individuals at high cancer risk who might be enrolled in chemotherapy trials is to identify those individuals who already display hallmarks of early stages of the carcinogenic process. Advances in mammography, endoscopy, and various types of imaging have increased the ability to detect early and sometimes preneoplastic lesions, for example adenomatous polyps of the colon, leukoplakia in the oral cavity, and Barrett's esophagus. Except for the PSA test for prostate cancer and α-fetoprotein for liver cancer, serum markers to detect early stages of cancer have not, in general, been useful. This approach merits further investigation. A promising approach is the use of highly sensitive immunologic and molecular genetic tools for identifying individuals who display preneoplastic or early neoplastic lesions, by detecting mutations or altered levels of expression of specific genes, or alterations in repetitive DNA sequences. This topic is discussed in greater detail in the paper by David Sidransky in this symposium.

With respect to the latter type of approach, there are now a plethora of genes that display mutations and/or altered expression in various types of human cancer [2–5]. Some of these changes might be exploited to identify individuals at high risk, and also as targets for chemoprevention. Because of the large number and diverse functions of these genes, I believe that the categories "oncogenes" and "tumor suppressor genes" are becoming antiquated, especially because they do not indicate the specific biochemical functions of the individual genes or consider the contexts within which they function. Table II presents a classification scheme which attempts to achieve this goal. The genes are divided into two broad functional categories: A) those that control intracellular regulatory circuitry, and B) those that influence the cell surface and extracellular functions. The first category is further divided into three subcategories. Subcategory 1 includes genes that are involved in the responses of cells to external growth factors. These genes encode the growth factors themselves, cellular receptors, coupling proteins and protein kinases that transduce information across the cytoplasm to the nucleus, and nuclear transcription factors that then increase or repress the expression of specific genes. Many of the so called oncogenes fit into this sub-category. Subcategory 2 includes genes that control the cell cycle, DNA replication, DNA repair and genomic stability; and subcategory 3 includes genes that control cell fate with respect to cellular differentiation or pro-

grammed cell death (apoptosis). Subcategory 2 includes the tumor suppressor genes Rb and p53. Recent studies on cyclins and cyclin-related genes and their abnormalities in cancer have rapidly expanded subcategory 2, and this subject is discussed in greater detail, below. With respect to subcategory 3, progress is being made in identifying abnormalities in genes that either enhance or inhibit apoptosis in cancer cells but very little is known about the specific genes responsible for the frequent impairments in differentiation in cancer cells. Category B includes genes that influence how the cell interacts with the extracellular matrix and/or neighboring cells. This includes genes that encode various cell surface proteins, cell adhesion molecules, extracellular proteases, and angiogenesis factors. Alterations in these genes are especially relevant to tumor cell invasion and metastasis.

TABLE I. Factors That Influence Cancer Risk*

1. Inheritance of Predisposing Genes
   a. Familial Cancer Syndromes (single gene)
   b. Polymorphisms in:
      1) Drug metabolizing enzymes
      2) ? DNA repair enzymes
      3) ? Proteins involved in cell proliferation and differentiation
2. Exogenous Factors
   a. Cigarette Smoke
   b. Occupational and environmental carcinogens
   c. Lifestyle factors
   d. Dietary factors
   e. Viruses, bacteria, parasites
3. Existence of pre-neoplastic lesions, i.e., leukoplakia, dysplasia, etc. (Can molecular genetics identify pre-preneoplastic lesions?)

*Note: 1) Importance of gene/environment interactions. 2) A single risk factor may not identify "high" risk individuals. 3) Importance of biomarkers and molecular epidemiology to more precisely identify these risk factors.

TABLE II. Categories of Genes Involved in Carcinogenesis

A. Intracellular Circuitry
   1. Agonist-induced signal transduction
   2. Cell cycle control, DNA replication and DNA repair
   3. Cell fate: differentiation, apoptosis
B. Cell Surface and Extracellular Functions: Adhesion molecules, proteases, angiogenesic factors, etc.

I should emphasize that 1) many of the above-mentioned gene products perform multiple functions (i.e., the p53 protein), 2) various pathways in the cell interact via complex networks, and 3) the function of a given gene product is often dependent on the context of the specific cell type in which it is expressed. Therefore, the classification scheme shown in Table II is an oversimplification and should not be considered rigid or absolute. Nevertheless, I think that it is much more informative than simply the terms "oncogenes" and "tumor suppressor genes." Obviously, extensive further studies are required to determine which of this multitude of genes will be useful for detecting abnormalities that will be useful in identifying individuals who are at high risk of developing malignant tumors, and thereby subjects who are most appropriate for chemoprevention or other types of intervention studies. Highly sensitive, specific and cost effective methods must also be developed for identifying such abnormalities in biologic fluids, cytology specimens, or readily obtained tissue biopsies.

RECENT STUDIES ON ABNORMALITIES IN CYCLIN D1 IN HUMAN CANCER

As discussed above, cancers often display abnormalities in genes that govern the responses of cells to external growth factors, since they encode the growth factors themselves, growth factor receptors, proteins involved in pathways of signal transduction in the cytoplasm, or nuclear transcription factors (Table I, A.1). In this sense they determine whether cells will be in a resting non-dividing "Go" state or whether they will enter the G1 phase of the cell cycle and thereby undergo cell replication and proliferation. It is becoming increasingly apparent that a separate set of cellular genes can also be targets during the multistage carcinogenic process [for review see 6,7]. These genes normally control later events in the cell cycle, particularly during the late G1 and early S phases (Table I, A.2). Aberrations in these genes can also perturb cellular proliferation and growth control. Moreover, they might also contribute to genomic instability, thereby enhancing tumor progression and tumor heterogeneity. Therefore, in the remainder of this paper I will briefly review recent studies in this area, emphasizing the gene cyclin D1.

As originally discovered in lower organisms, the orderly progression of dividing mammalian

cells through the G1, S and G2/M phases of the cell cycle is governed by a series of proteins called cyclins which exert their effects through specific cyclin-dependent protein kinases (Fig. 1) [6,7]. Mammalian cells have "checkpoints" at the G1/S and G2/M transitions which delay progress through the cell cycle to permit repair of damaged DNA and possibly other toxic events. The normal Rb gene, originally identified in hereditary retinoblastomas and frequently mutated in a variety of sporadic human tumors, acts as a negative inhibitor at the G1/S checkpoint (Fig. 1). The p53 tumor suppressor gene (Fig. 1) also plays a critical role in the G1/S checkpoint since cells that are defective in p53 fail to show G1/S arrest in response to DNA damage, presumably because they fail to induce the cyclin-dependent kinase inhibitor (CDI) $p21^{WAF1}$, and possibly other proteins, which inhibit G1 cyclin/CDK activity. The gene mutated in Ataxia Telangiectasia appears to play a critical role in the accumulation of p53 in response to DNA damage. Very little is known about the G2/M checkpoint in mammalian cells but it is conceivable that defects acting at this stage might contribute to the chromosomal anomalies often seen in malignant tumors.

Several cyclin genes have been identified in mammalian cells [6,7]. The G1 cyclins (D1–3 and E) are maximally expressed during G1 and

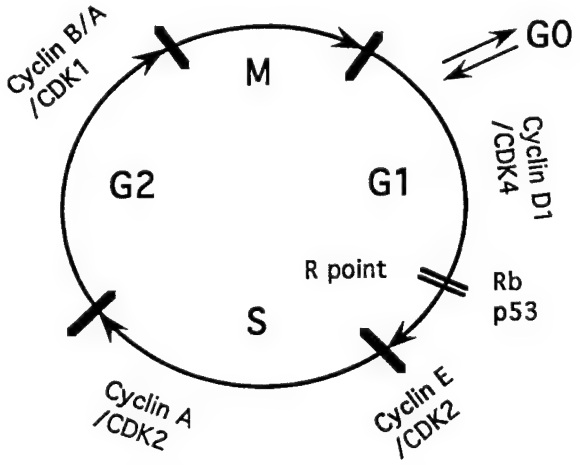

Fig. 1. A schematic diagram of the mammalian cell cycle indicating the G0 phase of nondividing cells, the G1 phase when cells enter the cell cycle and prepare for DNA synthesis, and the G2/M phase in which cells prepare for and undergo mitosis. Also shown are the cyclins and cyclin dependent protein kinases (CDKs) that act at specific phases of the cell cycle; and the restriction point "R," (also called the G1/S checkpoint), at which the Rb and p53 tumor suppressor genes can inhibit cell cycle progression. For additional details, see text and reference 6.

regulate progression of the cell cycle from mid-G1 into the S-phase. Cyclin A is highly expressed in early S-phase of the cell cycle and enhances progression through the S-phase. It also acts during the G2/M transition. Two B-type cyclins (B1 and B2) are important for the entry and exit of cells from mitosis. Four additional cyclins, cyclins C, F, G and H have been identified but their specific roles in cell cycle progression and tumorigenesis have not been studied in detail. Cyclins do not have their own enzymatic activity. Instead, they act by binding to and stimulating the activities of a series of cyclin-dependent protein kinases (CDK) [6,7]. The activities of these CDKs are regulated by phosphorylation on specific threonine and tyrosine residues, and by a group of specific inhibitory proteins called CDIs [6]. To date, at least eight mammalian CDKs have been identified [6]. CDK1 (also called Cdc2) is involved in regulation of the G2/M transition, in association with cyclin B. Cyclin A can also associate with CDK1 and this complex also plays a role in the G2/M transition. CDK2 is involved in regulating the G1/S transition and S phase progression by its association with cyclin E and cyclin A, respectively. CDK4 and CDK6 are the major catalytic partners for cyclins D1, D2 and D3, and these complexes can phosphorylate the retinoblastoma protein (pRb). D cyclins also complex with CDK5 but the function of these complexes are not known. Cyclin D1 can also complex with the DNA replication factor proliferating cell nuclear antigen (PCNA) and pRb. Several studies indicate that cyclin D1 is involved in inactivating the function of pRb, presumably through phosphorylation and/or the formation of a physical complex, thereby abrogating its inhibitory effect on G1/S progression (Fig. 1). When pRb is phosphorylated it no longer binds the transcription factor E2F. E2F can then act to turn on the expression of genes required for further cell cycle progression [5,6].

As mentioned above, several CDIs have been identified [6]. The protein $p21^{WAF1}$ (also called CIP1), whose synthesis is induced via the p53 protein in response to DNA damage, binds to various cyclin-CDK complexes, including cyclin D1-CDK4, cyclin D1-CDK6, cyclin E-CDK2, and cyclin A-CDK2, and inhibits their activation, thus causing cell cycle arrest. Similarly, the protein $p27^{Kip1}$ binds to the cyclin D1-CDK4, cyclin D1-CDK6, and cyclin E-CDK2 complexes and inactivates their function, thus arresting cells at G1/S. This occurs when cells undergo

contact-dependent inhibition of growth or inhibition of growth in response to treatment with the inhibitory growth factor TGF-β. It appears that a protein designated p15 (INK4B/MTS2) mediates this effect of TGF-β in human keratinocytes. The protein $p16^{INK4}$ (also called MTS1) binds to and inhibits the activity of CDK4 and CDK6. Additional CDIs have been recently identified. They include p18 and 19, which are related to p15 and $p16^{INK4}$; and p57, which is related to $p27^{KipI}$. Their precise normal functions, and possible abnormalities in cancer cells, remain to be determined.

There is increasing evidence that several types of human tumors display abnormalities in cyclin and cyclin-related genes [for review see 6,7]. There are numerous types of abnormalities in the cyclin D1 gene in human cancers. This gene, also termed prad 1 or *bcl*-1, is located at chromosome 11q13. Chromosomal rearrangements at this locus in parathyroid tumors, or centrocytic B cell lymphomas cause increased and constitutive expression of this gene. The cyclin D1 gene is amplified and overexpressed, at both the mRNA and protein levels, in a significant fraction of primary human breast carcinomas, esophageal carcinomas, squamous carcinomas of the head and neck, non-small-cell lung carcinomas, hepatocellular carcinomas, and bladder carcinomas. Cytogenetic and molecular studies indicate that the amplified cyclin D1 gene is part of a much larger amplicon located at chromosome 11q13. This amplicon can be as large as 1,000 kb and encompasses at least four additional genes. Overexpression of cyclin D1 in the absence of gene amplification is also seen in about 45% of human breast carcinomas [8] and about 40% of colon carcinomas [9,10], but the mechanisms responsible for this overexpression are not known.

Several types of mechanistic studies, specifically implicate the cyclin D1 gene in tumorigenesis. Thus, using gene transfer studies we found that stable overexpression of cyclin D1 in rodent fibroblasts enhanced their growth in cell culture and tumorigenicity in nude mice [11]. Co-transfection studies indicated that cyclin D1 cooperates with a defective adenovirus E1A gene [12] or an activated *ras* oncogene [13] in the transformation of rodent cell lines. Overexpression of a cyclin D1 sequence under the control of a MMTV promoter in transgenic mice resulted in mammary hyperplasia and tumors of the mammary epithelium [14], and cyclin D1 cooperated with the a *myc* oncogene in producing B cell lymphomas in transgenic mice [15,16]. Our laboratory has demonstrated that expression of an antisense cyclin D1 sequence in a human esophageal cancer cell line in which the endogenous cyclin D1 gene is amplified and overexpressed caused decreased levels of the endogenous cyclin D1 protein; reduction of in vitro cyclin D1-associated CDK protein kinase activity; marked inhibition of cell proliferation; and loss of tumorigenicity [17]. Thus, overexpression of cyclin D1 appears to play a critical role in both the establishment and maintenance of the transformed phenotype in certain types of human cancer. It is of interest that the cells that are reverted as a result of the antisense cyclin D1 sequence express a reduced but still relatively high level of cyclin D1, suggesting that the parental cells are "addicted" to cyclin D1, i.e., they require a very high level of this protein to maintain their tumorigenic phenotype [17].

In studies on human esophageal carcinomas we noted that the subset of tumors that had amplification and increased expression of cyclin D1 displayed normal expression of the Rb gene, whereas the subset of tumors that did not express the Rb protein (presumably due to deletion mutations) did not show amplification and increased expression of cyclin D1 [7]. Thus, it would appear that during the clonal evolution of tumors the inhibitory effect of the Rb gene on cell cycle progression can be abrogated, either by increased expression of cyclin D1, which would increase Rb phosphorylation of the Rb protein, thereby inactivating its inhibitory function, or actual loss of the Rb protein itself [7]. An alternative mechanism could be inactivation of CDIs that act on cyclin D1/CDK4 and cyclin D1/CDK6. These examples provide an explanation why different tumors of the same histologic type can differ with respect to their spectrum of gene mutations, since the same regulatory pathway can be perturbed in different tumors by mutations in different genes that influence this pathway. Therefore, in the design and use of new gene-specific anti-cancer agents it may be necessary to score individual tumors for the specific mutation involved or design agents that are pathway-specific rather than gene-specific.

Human tumors often display amplification and increased expression of several genes including cellular oncogenes and genes that confer drug resistance [2–4]. Therefore, gene ampli-

fication is an important cause of tumor progression and tumor heterogeneity. We have recently demonstrated that increased expression of cyclin D1 can enhance the process of gene amplification [18]. Therefore, cyclin D1 might play a critical role in the genomic instability often associated with tumor progression, and inhibition of the action of cyclin D1 might be a useful approach for blocking tumor progression [18].

The increased expression of cyclin D1 could be useful in identifying preneoplastic lesions in high risk individuals, since we have found that increased expression of cyclin D1 can be detected in adenomas of the colon, i.e., at a relatively early stage in the process of colon carcinogenesis [10] and also in Barrett's esophagus, a disease associated with an increased risk of esophageal cancer [19]. As discussed above, there is accumulating evidence that increased expression of cyclin D1 can enhance the conversion of normal cells to tumor cells and, by enhancing genomic instability, also accelerate the process of tumor progression. Furthermore, the overexpression of cyclin D1 is necessary for maintenance of the tumorigenic phenotype in some malignant cancer cells [17]. Taken together, these findings suggest that inhibitors of the action of cyclin D1 might be useful in both cancer chemoprevention and cancer therapy.

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Cell Cycle Control Gene Defects and Human Cancer

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GLOSSARY

amplification an increase in the number of copies of a gene at a specific locus.

cell cycle the set of events responsible for the duplication of the cell. It comprises a DNA synthesis phase (S), a mitotic phase (M), and two gap phases (G_1 and G_2) that are interposed between the M and S and S and M phases, respectively.

cyclin a family of proteins that bind to cyclin-dependent kinases (CDKs) and activate their kinase activity.

cyclin-dependent kinase (CDK) a family of serine/ threonine kinases that are activated by the binding of specific cyclins and whose sequential activation ensures the ordered progression of the cell cycle.

cyclin-dependent kinase inhibitor (CDI) a family of small molecular weight proteins able to associate with and inhibit the activities of CDKs.

genomic instability the ability of tumor cells to develop genetic variants by various types of mutational mechanisms at a greater frequency than normal cells.

G_1/S checkpoint the restriction point at late G_1 after which cells are insensitive to growth factor deprivation and commit themselves to another round of DNA replication.

p53 protein a transcription factor acting as tumor suppressor whose function is lost during the development of many types of tumors.

retinoblastoma (Rb) protein a nuclear phosphoprotein that suppresses cell proliferation through its inhibitory action on G_1/S transition.

Recent studies indicate that cell cycle regulatory proteins, mainly the cyclins and cyclin-related genes, can be critical targets during oncogenesis. These genes normally control specific events in the cell cycle, particularly during the late G_1 and early S phase. Aberrations in these genes can perturb cell cycle progression and cellular growth control. Moreover, they

might also contribute to genomic instability, thereby enhancing tumor progression and tumor heterogeneity.

I. INTRODUCTION

This article reviews current information on the roles of cell cycle regulatory proteins in the control of the mammalian cell cycle in normal cells and the types of abnormalities in these genes that have been found in human cancers. The possible clinical implications of these findings, with respect to cancer diagnosis, prevention, and treatment, are also discussed. The bibliography at the end of this article provides additional reviews of this subject and a few representative articles from the authors' research.

II. CELL CYCLE CONTROL IN NORMAL MAMMALIAN CELLS

The cell cycle is the set of events that is responsible for the duplication of the cell. It is typically subdivided into four phases: G_1, a phase during which the cell prepares to synthesize DNA; S, a period of DNA synthesis; G_2, a period in which preparations are made for cell division; and M, the mitotic phase itself. Cells in the body that are not actively dividing may be either terminally differentiated, thus unable to reenter the cell cycle, or in a state of temporary arrest called G_0. In addition, the cell cycle can be arrested at G_1/S or G_2/M "checkpoints." See Fig. 1 for a model of the cell cycle.

Ever since the 1970s there have been a series of discoveries which have provided a better understanding of the complexity of the control mechanisms which ensure ordered progression of the cell cycle. The eukaryotic cell cycle engine is composed of protein complexes that are activated in an ordered fashion. At the heart of this engine is a family of cyclin-dependent kinases (CDKs), which are regulated by a group of positive-activating partners called cyclins, and a family of negative inhibitors called cyclin-dependent kinase inhibitors (CDIs). In addition, CDKs are regulated by other kinases and phosphatases. These cyclin–CDK complexes that are formed and activated at specific stages of the cell cycle can then phosphorylate a number of target molecules, which carry out the steps that lead ultimately to DNA replication and cell division.

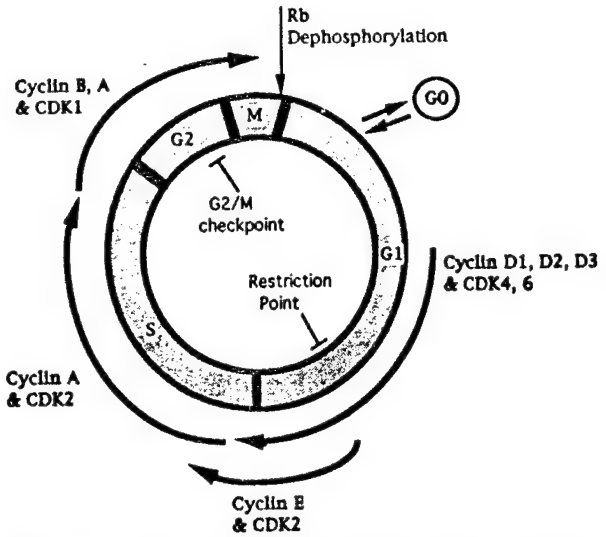

FIGURE 1 Simplified model of the cell cycle indicating the G_0 phase of nondividing cells; the G_1 phase when cells enter the cell cycle and prepare for DNA synthesis, which occurs in the S phase; and the G_2 and M phases in which cells prepare for and undergo mitosis. The major cyclin–CDK complexes acting at each phase of the cell cycle are also shown. There are two checkpoints during the cell cycle: the G_1/S checkpoint (also termed restriction point) at which Rb and p53 exert inhibition on the G_1/S transition, and the G_2/M checkpoint at which cells are prevented from progressing through the cell cycle. For additional details, see text.

A. Cyclin-Dependent Kinases

CDKs are a family of serine/threonine protein kinases. To ensure the proper timing and coordination of cell cycle events, CDK activity is tightly controlled by several complex mechanisms. Cellular CDK levels tend to remain in constant excess throughout the normal cell cycle, and the regulation of their catalytic activity is primarily posttranslational. The activation of CDKs requires the binding of an appropriate cyclin, whereas association with specific CDIs inhibits their activity. In addition, the activity of CDKs is positively regulated by phosphorylation of a conserved threonine (at residue 160, 161, or 172, depending on the specific CDK) by the CDK-activating kinase (CAK), which is itself the complex of cyclin H-CDK7, and dephosphorylation on a conserved threonine–

tyrosine pair (Thr-14–Tyr-15) by a series of CDC25 phosphatases. Conversely, phosphorylation of Thr-14–Tyr-15 by specific protein kinases and dephosphorylation of the active Thr site prevent the kinases from being activated. Figure 2 shows the multiple mechanisms regulating cyclin D1/CDK4 activity, which also apply to the regulation of other cyclin–CDK complexes.

Mammalian cells contain multiple CDK genes, including CDK1 (also termed CDC2 in yeast), and CDK2 to CDK8. Different cyclin–CDK complexes are assembled and activated at specific points to regulate progression through the G_1, S, G_2, and M phases of the cell cycle. Current evidence indicates that cyclin D (D1, D2, or D3) complexed to CDK4 or CDK6 regulates the transition in mid to late G_1. The cyclin E–CDK2 complex regulates the G_1–S transition, and the cyclin A–CDK2 complex is needed for progression through the S phase. Cyclin A–CDK1 is required for the G_2–M transition, but cyclin B–CDK1 is the main effector at this step. Figure 1 shows the major cyclin–CDK complexes that act at each phase of the cell cycle.

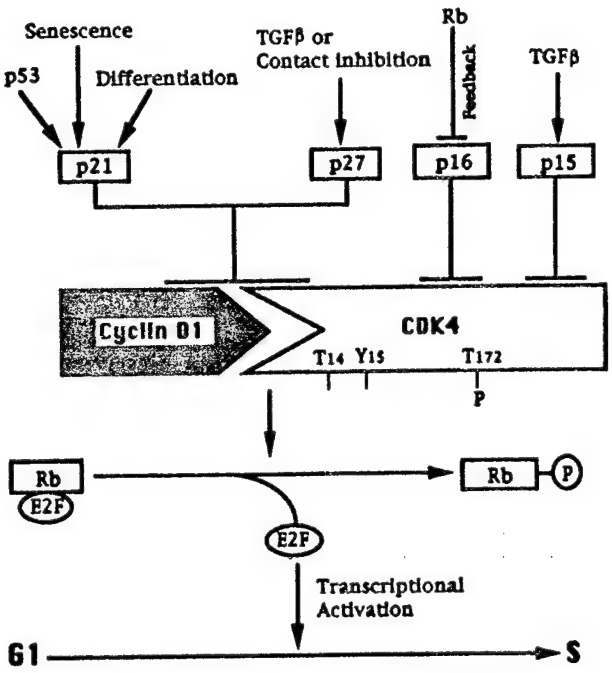

FIGURE 2 Multiple mechanisms regulate cyclin D1–CDK4 activity. The binding of cyclin D1 can activate the kinase activity of CDK4. Phosphorylation on a conserved Thr (Thr-172 in CDK4) and dephosphorylation on Thr-14 and Tyr-15 are required for activation of the complex. A group of inhibitory proteins (CDIs) designated $p21^{CIP1}$, $p27^{KIP1}$, $p16^{INK4}$, p15, and other related proteins bind to the cyclin–CDK complex and inhibit kinase activity. Various external factors acting through the CDIs can cause cell cycle arrest. Decreased Rb expression induces $p16^{INK4}$. Phosphorylation of the Rb protein by the active cyclin D–CDK4 complex can release E2F transcription factors, which enhances the G_1 to S transition and the onset of DNA synthesis in the S phase. For additional details, see text.

B. Cyclins

Cyclins were first identified in marine invertebrates as proteins that accumulated at high levels following the fertilization of eggs and then underwent abrupt destruction during mitosis. Since then, over 30 cyclin sequences have been identified from a variety of eukaryotic organisms, from yeast to human. Based on their conserved sequence motifs with other species, patterns of expression, and functional roles during the cell cycle in mammalian cells, they fall into several categories: (1) G_1 cyclins, including cyclin C, D1, D2, D3, and E; (2) a S-phase A-type cyclin; (3) two G_2/M phase B-type cyclins B1 and B2; (4) cyclin H which associates with CDK7 to form CAK which phosphorylates and activates CDK1, CDK2, and CDK4; (5) cyclin G which is a novel transcriptional target of the p53 tumor suppressor gene product and is induced after DNA damage; and (6) two cyclins with unknown function, cyclin F and X.

The best and first characterized cyclins are B-type cyclins, which have been found in all eukaryotes. They associate with the CDK1 subunit and the complexes show maximal protein kinase activity during metaphase. Cyclin B is first synthesized during the S phase, accumulates in complexes with CDK1 as cells approach the G_2/M transition, and is then abruptly degraded during mitosis. Phosphorylation of CDK1 on Thr-161 may stabilize its binding to cyclin B and is required for the subsequent activation of the enzyme. Other phosphorylations on Thr-14 and Tyr-15 within the CDK1 ATP-binding site maintain the kinase in an inactive form throughout S and G_2. Removal of the inhibitory phosphates from cyclin B-associated CDK1 at the G_2/M transition by CDC25 phosphatase activates the kinase and triggers entry into mitosis. Conversely, the exit from mitosis depends on the abrupt ubiquitin-mediated degradation of cyclin B during anaphase, resulting in the release of CDK1 as an inactive monomer.

Cyclin A can also interact with CDK1 to induce mitosis. However, the synthesis and destruction of cyclin A oscillate in advance of cyclin B. The synthesis of cyclin A is initiated during late G_1 and its associated kinase activity is first detected in the S phase. Cyclin A binds to at least two CDKs depending on the phase in the cell cycle: a cyclin A–CDK1 complex is involved in the G_2/M transition, and a cyclin A–CDK2 complex is involved in the G_1/S transition and S phase. Microinjection of antibodies to cyclin A or antisense cDNAs to cyclin A results in the blockage of entry into the S phase and inhibition of DNA synthesis, providing evidence that it is required for DNA replication. In agreement with this, cyclin A colocalizes with sites of DNA replication in S-phase nuclei, indicating that the cyclin A–CDK2 complex may have DNA replication proteins as targets.

Mammalian cells must respond to extracellular growth factors, mitogenic antagonists, and differentiation inducers in exercising their commitment to enter the S phase. The cells are sensitive to such stimuli until they reach a restriction point late in G_1, at which the cells are committed to another round of DNA replication. Key factors that control G_1 progression in mammalian cells include three D-type cyclins (D1, D2, and D3), which assemble into holoenzymes with either CDK4 or CDK6, and cyclin E, which combines later in G_1 with CDK2.

Cyclin E expression is periodic and peaks sharply at the G_1/S transition. It assembles with CDK2 and induces maximal levels of cyclin E-dependent kinase activity at this transition, suggesting that it controls the ability of cells to enter the S phase. Once cells enter the S phase, cyclin E is degraded and CDK2 forms complexes with cyclin A. Both complexes are thought to be essential for initiating DNA replication. Ectopic overexpression of cyclin E in mammalian fibroblasts shortens the G1 interval, decreases cell size, and reduces the serum requirement for G_1/S transition, thus causing premature entry into the S phase. However, the doubling time of cells overexpressing cyclin E is unchanged, and the contracted G_1 interval is balanced by a compensatory prolongation of both the S and G_2 phases. Perhaps the premature entry into the S phase limits the accumulation of factors required for DNA replication. Conversely, the microinjection of antibodies against cyclin E in mammalian fibroblasts prevents S-phase initiation. These results demonstrate that cyclin E synthesis is one of the rate-limiting steps for the G_1/S transition in mammalian cells.

C. Cyclin D1

D-type cyclins are rate-limiting controllers of the G_1 phase progression in mammalian cells. The three D-type cyclins (D1, D2, and D3) are differentially expressed in various cell lineages, implying that they are not functionally redundant. When growth factor-deprived cells are restimulated to enter the cell cycle, D-type cyclins peak in mid to late G_1 earlier than cyclin E. However, in continuously proliferating cells, the D cyclins are expressed throughout the cell cycle and their levels oscillate only minimally. Because cyclin D mRNAs and proteins turn over rapidly (with a half-live of about 20 min), removal of mitogens in G_1 results in their rapid degradation and the failure of cells to enter the S phase, but their destruction later in the cell cycle is without effect. Interference with cyclin D1 function by the microinjection of antisense plasmids or antibodies into normal fibroblasts or tumor cells during G_1 prevents cells from entry into the S phase, but injection near the G_1/S transition does not. This suggests that cyclin D-dependent kinases execute critical functions during mid to late G_1 phase, as cells cross the G_1 restriction point after which they become independent of mitogens for completion of the division cycle. Furthermore, the overexpression of cyclin D1 leads to a shortened duration of the G_1 phase, decreased cell size, and reduced serum dependency, and partially overrides transforming growth factor β (TGFβ) inhibition of growth in human esophageal epithelial cells. Therefore, deregulated expression of the cyclin D1 gene can cause disturbances in cell cycle control and normal mitogenic signaling pathways, which may contribute to its ability to enhance cell transformation and tumorigenicity.

Cyclin D1 associates with several CDKs, including CDKs 1, 2, 4, 5, and 6, but thus far only the complexes of cyclin D1–CDK4 and cyclin D1–CDK6 display enzymatic activities. Although cyclin D synthesis begins during the G_0 to G_1 transition, the associated kinase activity is not manifested until mid-G1 and

increases as cells approach the G_1/S boundary. The holoenzymes of cyclin D–CDK4/6 have a distinct substrate preference for the retinoblastoma protein (Rb), which functions as a growth inhibitor in the late G_1 phase of the cell cycle, rather than histone (H1), which is the major *in vitro* substrate for the other CDKs. In addition, cyclin D1 binds directly with Rb *in vitro* and *in vivo*. However, fibroblasts engineered to overexpress both cyclin D and CDK4 ectopically can still be rendered quiescent because the constitutively synthesized subunits do not assemble into active kinases in serum-deprived cells. This suggests that mitogenic signaling is still required to facilitate the formation of an active cyclin D–CDK complexes.

Although binary cyclin D–CDK complexes are fully capable of phosphorylating their substrates *in vitro*, the holoenzymes isolated from proliferating mammalian cells copurify with several other proteins, including proliferating cell nuclear antigen (PCNA) and the 21-kDa polypeptide $p21^{CIP1}$, which belongs to a family of small cyclin-dependent kinase inhibitor proteins (CDIs). PCNA is an auxiliary subunit of DNA polymerase δ required for DNA replication and repair. In primary fibroblasts, downregulation of cyclin D1 is necessary for PCNA relocation to nuclear structures in order to facilitate DNA repair or replication. Conversely, acute overexpression of cyclin D1 prevents the cells from entering the S phase and from repairing their damaged DNA; this effect is abolished by coexpression of PCNA. Moreover, the increased expression of cyclin D1 in certain mammary epithelial cell lines can also inhibit growth and enhance apoptosis. These data suggest that, depending on the specific cell context, cyclin D1 may also play a role in mechanisms that inhibit cell growth.

The other protein in the cyclin D1–CDK4 complex is $p21^{CIP1}$, a p53-inducible inhibitor of cyclin–CDKs that facilitates G_1 arrest in response to DNA damage or other stresses. Thus, by inhibiting cyclin–CDK kinase activity, p53 is able to cause cell cycle arrest.

D. Cyclin-Dependent Kinase Inhibitors

A new mechanism for the regulation of CDKs was discovered in the form of proteins that bind to and inhibit the active kinase complexes. These cyclin–CDK inhibitors (CDIs) provide fundamental mechanisms underlying negative growth control and checkpoint function, and their loss or inactivation could lead to the uncontrolled proliferation found in cancer cells. The mammalian CDIs fall into two categories based on their structural homology and functional similarity. One category includes $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$. These Cip/Kip inhibitors have broad specificity. They bind to and inhibit the kinase activity of the G_1-phase cyclin D–CDK4/6 and cyclin E–CDK2; the S-phase cyclin A–CDK2; and, to a lesser extent, the mitotic cyclin B–CDK1. Cip/Kip inhibitors cause G_1 arrest when overexpressed in transfected cells, and overexpression of the conserved CDK-binding/inhibitory domain from these inhibitors is sufficient for this G_1 arrest. The second category of mammalian CDIs includes $p16^{Ink4}$, $p15^{Ink4b}$, $p18^{Ink4c}$, and p19. All of these proteins contain a fourfold repeated ankyrin-like sequence and show no structural similarities to the Cip/Kip family. Ink4 inhibitors are specific for CDK4 and CDK6, and compete with cyclin D for binding to these kinases. As with the Cip/Kip inhibitors, overexpression of Ink4 inhibitors also blocks cellular proliferation. Figure 2 provides a scheme showing the functions of various CDIs in integrating several external signals into cell cycle regulation, focusing on G_1- and S-phase events.

The first mammalian CDI to be identified was p21 (also known as WAF1, CIP1, SDI1, and CAP20). The p21 protein has at least two separate roles: as a cyclin–CDK inhibitor through its binding to the kinase complexes and as a PCNA inhibitor via binding to PCNA and inhibiting processive PCNA-dependent DNA replication, without interfering with PCNA-dependent nucleotide excision repair. Its expression is induced in senescent cells and is capable of blocking growing cells from entering the S phase by transient transfection. In addition, its transcription is turned up by p53 after DNA damage, whereas in p53 negative cells from Li–Fraumeni patients, p21 is absent from cyclin–CDK complexes. However, p21 can also be regulated in a p53-independent manner. Its expression correlates with terminal differentiation in several lineages, both during embryogenesis and *in vitro* myoblast differentiation. Thus, p21 functions in many ways. In proliferating cells, it can arrest cells in G_1; in S-phase cells, it can, theoretically, block PCNA and

slow down DNA synthesis to facilitate DNA repair; and, in development, it may contribute to exit from the cell cycle during terminal differentiation.

$p27^{Kip1}$ was first noticed as a cyclin E–CDK2 inhibitor that binds to this complex in TGFβ-treated or contact-inhibited cells. Its expression is high under these conditions, as well as in serum-deprived cells, and declines after the mitogenic stimulation of resting cells. However, antiproliferative agents such as cAMP prevent mitogen-induced p27 downregulation in macrophages.

The $p16^{INK4}$ protein was identified by its association with human CDK4 in a yeast two-hybrid protein interaction screen. It binds to CDK4 and blocks the association of cyclin D1 and CDK4. The expresssion of p16 seems to be negatively regulated by the functional pRb protein, and the inhibitory effect of p16 on cell cycle progression is Rb dependent. Therefore, in cell lines lacking pRb, high levels of p16 are observed, and overexpresssion of p16 does not suppress the growth of these cells.

CDIs are capable of inhibiting growth. Several lines of evidence suggest that at least some CDIs may be tumor suppressor proteins and that deregulation of these proteins can be involved in tumor development (see later).

III. CELL CYCLE REGULATORS AND CANCER

Studies on the functions of cellular protooncogenes and tumor suppressor genes indicate that most of these genes mediate signal transduction pathways as well as cell cycle controls that play a critical role in cell proliferation and differentiation . This has led to the realization that cell cycle regulatory proteins can also be directly involved in oncogenesis. Recent developments have also deepened our understanding of the role played by cell cycle components in regulating the known tumor suppressors, pRb and p53 proteins.

The current model of cell cycle control is that the transitions between different phases of the cell cycle are regulated at specific checkpoints. One of the most important checkpoints is the restriction point in late G_1, at which the cell commits itself to another round of DNA replication and at which both positive and negative external signals are integrated into the cell cycle. This checkpoint is often deregulated in tumor cells due to changes in the sequential activation of specific cyclin–CDK complexes. This deregulation can result either from overexpression of the positive regulators, such as the cyclins and CDKs, or from the loss of the negative regulators, such as CDIs.

Table I is a summary of the major cyclin and cyclin-related abnormalities in human cancers that have been seen thus far. It is likely that additional abnormalities will also be discovered.

A. Cyclins and Cancer

A large body of data implicates cyclins in oncogenesis. The first evidence came from studies of human cyclin A. The cyclin A gene was found to be the unique insertion site of a hepatitis B virus (HBV) in one clonal hepatoma. This integration event results in a chimeric protein: the amino-terminal region of cyclin A is replaced by sequences from the pre-S_2/S protein of HBV. The cyclin A degradation sequence, located at the N terminal, is therefore deleted and the HBV–cyclin A chimeric protein is not degraded in normal fashion during mitosis. Because the activation of CDKs by binding to cyclin A is required in both S-phase progression and the G_2/M transition, the absence of degradation of cyclin A might lead to unregulated and premature DNA synthesis and to cell proliferation, thus contributing to cell transformation. Nevertheless, changes in cyclin A expression appear to be rare in most types of cancer. Cyclin A is also of interest to tumorigenesis because it coprecipitates with the E1A protein in adenovirus-infected cells, suggesting that cyclin A might be involved in the adenovirus-induced cell transformation. In addition, deregulation of cyclin A synthesis could be involved in the anchorage-independent growth properties of transformed cells because fibroblasts engineered to overexpress cyclin A are able to grow in agar suspension.

There is often increased and/or deregulated expression of the cyclin E gene in human breast tumor cell lines and in primary human breast, colon, and prostate carcinomas, but, with rare exceptions, it is not usually amplified. Human tumors often express a series of lower molecular weight forms of the cyclin E proteins,

TABLE I

Abnormalities in Cell Cycle-Related Genes in Human Cancers[a]

Gene	Type of abnormality	Type of cancer
Cyclin		
Cyclin A	Site of integration of hepatitis B virus, yielding a stabilized protein	Liver
Cyclin E	Increased expression and aberrant low molecular weight forms	Breast, colon, prostate
Cyclin D1	Chromosomal translocations cause constitutive high expression	Parathyroid (PRAD1) B-cell lymphoma (BCL1)
	Gene amplification and increased expression	Breast, esophagus, head and neck, lung, liver, bladder
	Increased expression without amplification	Breast, colon
Cyclin-dependent kinase		
CDK4	Amplification and increased expression	Gliomas, sarcomas
	Mutation that disrupts $p16^{INK4}$ binding	Melanoma
Phosphatase		
CDC25B	Increased expression	Breast
Cyclin-dependent kinase inhibitor		
$p16^{INK4}$	Loss of expression due to deletions, point mutations, or DNA methylation	Pancreas, esophagus, gliomas, leukemias, etc.
$p15^{INK4b}$	Deletions (linked to $p16^{INK4}$ gene)	Leukemias, lung, etc.
$p21^{CIP1}$	Impaired induction after DNA damage in p53 mutant cells	Numerous types of cancer
$p27^{KIP1}$	Impaired function in TGFβ-resistant cells	Numerous types of cancer
G1/S checkpoint control proteins		
p53	Loss of heterozygosity, point mutations, and inactivation by viral oncoproteins	Numerous types of cancer
Rb	Loss of heterozygosity, deletions, point mutations, and inactivation by viral oncoproteins	Numerous types of cancer

[a]Note that many tumors display dysregulation in the expression of cyclin D1, E, and A. For additional details, see text.

but the reason for this and its functional significance remains to be identified.

B. Cyclin D1 and Cancer

Because the major regulatory events leading to mammalian cell proliferation and differentiation occur in the G_1 phase of the cell cycle, deregulated expression of the G_1 cyclins and CDKs might cause loss of cell cycle control and thus enhance oncogenesis. Indeed, the strongest connection between cyclins and oncogenesis comes from studies on cyclin D1. Cyclin D1, originally isolated as a gene located on human chromosome 11q13, is rearranged in a subset of parathyroid adenomas (referred to as PRAD1). This rearrangement fuses the 5′ regulatory region of the parathyroid hormone (PTH) gene to DNA immediately upstream of the PRAD1 (cyclin D1) gene promoter, resulting in a dramatic increase in PRAD1 mRNA and protein. Presumably, the deregulated overexpression of the PRAD1/cyclin D1 gene contributes to the tumorigenicity of these cells. The chromosome region 11q13 is also frequently the site of reciprocal translocations t(11;14)(q13;q32) in certain B-cell centrocytic lymphoma and leukemias. The region of translocation, termed BCL-1, was assumed to contain an unknown protooncogene that could be activated by this translocation-mediated juxtaposition with the immunoglobulin heavy chain enhancer element normally located on 14q32. Later on, genomic mapping associated with biochemical studies identified that cyclin D1 was the BCL-1 protooncogene. All of these translocations leave the cyclin D1 encoding exons intact and are uniformly associated with overexpression of cyclin D1 mRNA and protein, both in primary human tumors and in cell lines carrying the t(11;14).

Human chromosome band 11q13, to which cyclin D1 gene is assigned, is also involved in tumor-specific DNA amplification events in several types of human tumors. The cyclin D1 gene is amplified and overexpressed, at both the mRNA and protein levels, in a significant fraction of primary human breast carcinomas, esophageal carcinomas, squamous carcinomas of the head and neck, non-small cell lung carcinomas, hepatocellular carcinomas, and bladder carcinomas. The presence of amplification at 11q13 has been related to a shortened survival and undifferentiated cancers. In addition, increased expression of cyclin D1, in the absence of gene amplification, also occurs in about 45% of human breast carcinomas and in about 40% of human colon carcinomas. The mechanisms responsible for this increased expression are not fully determined yet. An interesting example in one breast carcinoma cell line is that cyclin D1 is overexpressed because its mRNA is stabilized as a consequence of truncation of the 3′-untranslated region.

Overexpression of cyclin D1 in rat fibroblasts enhances their growth and induces tumors in nude mice, although it is not sufficient to transform primary mammalian cultures. However, protooncogenes, such as *myc* and *ras*, are able to cooperate with cyclin D1 in transforming cells. For example, cyclin D1 cooperates with an activated *ras* oncogene to transform rat embryonic fibroblasts and cooperates with *myc* to induce B-cell lymphomas in transgenic mice. Furthermore, overexpression of cyclin D1 under the control of the mouse mammary tumor virus long terminal repeat in transgenic mice results in increased mammary cell proliferation and in the development of mammary tumors.

Overexpression of cyclin D1 plays a role not only in establishing the transformed phenotype, but also in maintaining the transformation. Thus, we found that expression of an antisense cyclin D1 cDNA construct in a human esophageal carcinoma cell line, in which the cyclin D1 gene is amplified and overexpressed, reduced the level of the cyclin D1 protein and caused decreased cyclin D1-associated kinase activity, marked inhibition of cell proliferation, and loss of tumorigenicity in nude mice. Because the 11q13 amplicon where cyclin D1 resides spans over 1000 kb and encompasses several other putative protooncogenes, this study also provides direct evidence that cyclin D1 is the responsible oncogene in this large amplicon. The ability to revert the transformed phenotype of these cells with antisense cyclin D1 suggests that cyclin D1 or cyclin D1–CDK4 kinase activity might be useful targets in cancer therapy. Consistent with this idea is the finding that microinjection of a cyclin D1 antibody into tumor cells in which cyclin D1 is amplified blocks the cells in the G_1 phase. In addition, cyclin D1 knockout mice show impaired retinal development during embryogenesis and fail to undergo the usual extensive proliferation of mammary epithelium associated with pregnancy.

Defects in cell cycle control and increased genomic instability, including gene amplification, often occur during cancer development. We have demonstrated that the overexpression of cyclin D1 in a rat epithelial cell line is able to enhance PALA [*N*-(phosphonacetyl)-L-aspartate]-induced amplification of the CAD (carbamyl synthetase, aspartate transcarbamylase, and dihydrooratase) gene by over 1000-fold. This acquired ability to amplify DNA in the cyclin D1-overexpressing cells correlates with their loss of G_1/S checkpoint control. The capacity of cyclin D1 to enhance genomic instability may also contribute to its oncogenic activity.

The cyclin D2 gene was mapped to human chromosome 12p13. The corresponding locus on mouse chromosome 6 was identified as vin-1, the site of integration of a murine leukemia provirus in a mouse T-cell leukemia, which resulted in cyclin D2 overexpression. Cyclin D2 amplification was noted in a colorectal carcinoma cell line, but this appears to be a rare event. The cyclin D3 gene was mapped to chromosome 6p21. Thus far, it has not been implicated in human cancer. The short arms of both chromosomes 12 and 6 are involved in cytogenetic abnormalities in various human tumors, suggesting that increased gene dosage might activate the respective cyclins and contribute to tumorigenesis.

The association between D-type cyclins and tumorigenesis is strengthened by the compelling evidence that these cyclins exert their effects on cell cycle regulation through the retinoblastoma tumor suppressor protein, pRb. pRb is a 105- to 110-kDa nuclear phosphoprotein ubiquitously expressed in mammalian tissues. It was first identified as the gene mutated in hereditary human retinoblastomas, but somatic mutations frequently occur in a variety of other more common tumors. There is also evidence

that pRb normally acts as a tumor suppressor since it is capable of preventing or suppressing cell division in certain cell types. The ability of oncoproteins encoded by several DNA tumor viruses to bind and inactivate Rb protein further suggests the importance of pRb as a negative regulator of S-phase entry. The pRb protein functions by association with a variety of cellular proteins, most notably the E2F family of transcription factors (see Fig. 2). Binding of pRb with E2F prevents the ability of E2F to activate the transcription of various genes, including genes involved in the onset of DNA synthesis and S-phase progression, such as c-*myc*, cyclin A, dihydrofolate reductase, and thymidylate synthetase.

The tumor suppression ability of pRb is regulated by cell cycle-dependent phosphorylation. pRb is underphosphorylated throughout the early and mid-G_1 phase, phosphorylated in late G_1, and remains phosphorylated until late mitosis. The hypophosphorylated form of pRb is active in E2F association and can suppress growth in G_1, whereas phosphorylation of pRb relieves the E2F binding and inhibition. The initial phosphorylation of pRb during mid to late G_1 is most probably carried out by cyclin D-dependent kinases, which are active during this interval. Cyclin D–CDK4 complexes can phosphorylate pRb *in vitro*. Cyclin D1–CDK4 phosphorylates most of the sites that are phosphorylated *in vivo* when Rb is phosphorylated in late G_1. Moreover, cyclin D binds directly to hypophosphorylated forms of pRb, through an N-terminal LXCXE motif shared by cyclin D and Rb-binding oncoproteins, and this physical association may help to direct CDK4 to its substrate during the G_1 phase.

The functions of cyclin D1 and Rb appear to be interdependent. Overexpression of cyclin D1 accelerates Rb phosphorylation and G_1 progression. Cyclin D1 is dispensable for G_1 control in Rb gene-deficient cells because the microinjection of cyclin D1 antibodies into these cells does not affect cell cycle progression, indicating that the major role of cyclin D1 is to inactivate Rb so that cells can enter the S phase and replicate their DNA. Also, it has been found that the levels of cyclin D1 protein and cyclin D1–CDK4 complexes are significantly downregulated in Rb minus cells, and ectopic expression of Rb induces cyclin D1, suggesting that cyclin D1 synthesis and activation leading to Rb phosphorylation would form a negative feedback loop in late G_1 which would turn down cyclin D1 expression.

C. CDKs, CDC25 Phosphatases, and Cancer

In addition to abnormalities in cyclin genes, there are also documented examples of abnormalities in human cancers in CDK genes and in CDC25 genes whose protein products modulate the state of phosphorylation of CDK proteins. The catalytic subunits of cyclin D1, the CDK4 and CDK6 proteins, have been found to be targets for aberrant changes during tumorigenesis. Both of these CDKs are overexpressed in several tumor cell lines, and about 10–30% of gliomas and sarcomas display amplification and increased expression of the CDK4 gene. Because the amplified CDK4 gene in these tumors is part of a much larger amplicon located at chromosome 12q13, it is possible that other amplified genes in this region, such as MDM2, GLI, and SAS, also contribute to tumorigenesis. A $p16^{INK4}$-insensitive CDK4 mutant has been found in some human melanomas. This mutation of CDK4 creates a tumor-specific antigen and can disrupt cell cycle regulation exerted by the cyclin-dependent kinase inhibitor $p16^{INK4}$.

As discussed earlier, the kinase activity of CDKs can also be inhibited through phosphorylation on the conserved residues, Thr-14 and Tyr-15. During the cell cycle, a series of CDC25 phosphatases dephosphorylate these residues, thus activating the respective CDKs. Human cells have three CDC25 genes, designated A, B, and C. Transfection experiments indicate that CDC25A or B, but not C, cooperate with either an activated *ras* oncogene or loss of Rb in transforming murine fibroblasts. Several tumor cell lines and about 30% of human primary breast cancers display an increased expression of CDC25B, suggesting that the increased expression of CDC25 phosphatases may contribute to the development of human cancer.

D. CDIs and Cancer

CDK inhibitors (CDIs) provide another set of fundamental mechanisms of tumor suppression. Because loss of negative growth control and normal checkpoint function are common features of tumor cells,

the deregulation of CDIs may be involved in tumor development.

Among the CDIs, the most notable candidate for a role as a tumor suppressor is $p16^{INK4}$, which is a specific inhibitor of cyclin D–CDK4/6. The human p16 gene lies on chromosome 9p21. The p16 gene locus is rearranged, deleted, or mutated in a majority of tumor cell lines, leading to the suggestion that p16 corresponds to the multiple tumor suppressor gene (MTS1) that maps to this chromosome region, whose inactivation has been implicated in tumorigenesis in a wide variety of cancers, including sporadic carcinomas and familial melanomas (MLMs). As many as 70% of tumor cell lines from various tumor types contain alterations at the p16 locus. However, the significance of p16 mutations in primary tumors is still disputed, as they occur with a much smaller frequency than in cell lines. Nevertheless, several studies have shown loss of p16 expression in familial melanomas, esophageal squamous cell carcinomas, glioblastomas, leukemias, lung, bladder, and pancreatic carcinomas. Furthermore, this gene can be inactivated by several mechanisms, including gross deletions, various types of point mutations, and DNA methylation. Transfection experiments demonstrate that p16 can act as a potent and specific inhibitor of progression through the G_1 phase, and several tumor-derived alleles encode functionally compromised proteins. The ability of p16 to induce cell cycle arrest is lost in cells lacking functional Rb, supporting the notion that overexpression of cyclin D1, loss of p16, and loss of Rb have similar effects on G1 progression and may represent a common pathway to tumorigenesis. It is of interest that the chromosomal locus encoding p16, named *INK4a*, also gives rise to another distinct transcript from an alternative reading frame which encodes a protein designated p19. The p19 protein has the ability to arrest cell proliferation at both G_1 and G_2. Mice carrying a targeted deletion of the *INK4a* locus, which eliminates the expression of both p16 and p19 develop spontaneous tumors at an early age. Therefore, it appears that the *INK4a* locus normally functions as a tumor suppressor.

The $p15^{INK4b}$ (also termed MTS2) gene, which is adjacent to the p16 locus, encodes a very similar protein and is a potential effector of TGFβ-induced G_1 arrest. It is also a putative tumor suppressor gene because the majority of homozygous deletions that remove p16 in cell lines also remove p15.

Strong evidence for a role in cellular transformation has been obtained for another CDI, the p21 protein. p21 inhibits a variety of cyclin–CDK complexes, including cyclin D–CDK4/6 and cyclin E–CDK2, which are strongly implicated in regulating the G_1/S checkpoint, and cyclin A-CDK2, which appears to be required for ongoing DNA replication. The link between p53 and p21 suggests that p21 can be an important mediator of p53-dependent tumor suppression. Activation of p53 after DNA damage induces p21 expression, thus leading to the inhibition of cyclin–CDK activities and preventing cells from entering the S phase. Conversely, in cells lacking p53, the failure to induce p21 after DNA damage might allow the replication of damaged DNA and contribute to the increased incidence of chromosomal abnormalities and genetic instability in transformed cells. In addition, the expression of p21 is increased during skeletal muscle differentiation, and overexpression of cyclin D1 can inhibit myogenic differentiation in proliferating myoblasts, suggesting that the p21 protein may function during development as an inducible growth inhibitor that contributes to cell cycle exit and differentiation. Thus, loss of p21 may disturb the normal differentiation pathway during tumor development, thereby contributing to tumorigenesis. However, data indicate that p21 knockout mice (unlike p53-/- mice) do not appear to display an increase in spontaneous malignancies and that the deletion of p21 has no apparent effect on intestinal cell lineage differentiation. Nevertheless, the p21-deficient cells are significantly defective in their ability to arrest in G_1 after DNA damage. These findings suggest that p21 does play a critical role in G_1 checkpoint control, but that the anti-oncogenic effects of p53 appear to involve proteins in addition to p21.

p27 is another universal cyclin–CDK inhibitor. The fact that p27 is activated from a sequestered state by TGFβ treatment or by contact inhibition suggests that it plays a role in the TGFβ-mediated inhibition of Rb phosphorylation and growth arrest. Transformed cells often display impaired contact inhibition of growth and fail to arrest in response to TGFβ treatment, suggesting defects in the function of p27 in oncogenesis. Furthermore, mice that are homozygous

for a targeted deletion in the p27 gene and therefore do not express the p27 protein display an overall increase in body size, hyperplasia of multiple organs, female sterility, and pituitary tumors, thus providing further evidence that p27 normally inhibits growth. However, mutations in the p27 gene itself have not been seen thus far in cancer.

Collectively, the CDIs are extremely important molecules in cancer biology. Their biochemical functions and their patterns of expression imply roles in the regulation of important steps in cell growth and differentiation. However, as mentioned earlier, mutations have only been seen thus far in the p16 gene in cancer. It seems likely, therefore, that the growth control pathway involving cyclin D1, CDK4/6, and p16 may be of particular importance for tumorigenesis.

IV. CLINICAL RELEVANCE

It is apparent from the earlier discussion and the summary in Table I that numerous abnormalities in cyclins and cyclin-related genes occur in human cancers. Indeed, one or more of these abnormalities may be present in the majority and perhaps all forms of human cancer. Abrogation of the G_1/S checkpoint has emerged as a frequent theme in the clonal evolution of cancer, either through the impairment of negative acting factors that normally inhibit cell cycle progression, such as Rb, p53, $p16^{INK4}$, $p15^{INK4b}$, $p21^{CIP1}$, and $p27^{KIP1}$, or through the increased function of positive acting factors, including cyclin D1, cyclin E, CDK4, and CDC25B.

The ubiquitous nature of these changes in human cancer suggests that they might be exploited for improving cancer diagnosis, prevention, and treatment. The increased expression of cyclin D1 can be detected by immunohistochemistry in certain precursor lesions, e.g., Barrett's disease of the esophagus and adenomatous polyps of the colon. Thus, it might serve as a useful biomarker for diagnostic studies. In addition, the fact that an increased expression of cyclin D1 can occur at an early stage in the multistage carcinogenic process suggests that cyclin D1 or cyclin D1/CDK4 activity are potential targets for cancer chemoprevention. Evidence shows that amplification of the 11q13 region, which contains the cyclin D1 gene, is a marker of poor prognosis in squamous carcinomas of the esophagus. Further studies are required to determine whether the increased expression of cyclin D1 or some of the other abnormalities listed in Table I can be used as markers of prognosis or guides to therapy for other types of human cancer.

As discussed earlier, we have demonstrated that an antisense construct to cyclin D1 caused phenotypic reversion and inhibition of tumorigenesis in a human esophageal cancer cell line. These findings suggest that drugs designed to inhibit the action of cyclin D1 or cyclin D1/CDK4 might be useful in cancer therapy, especially for tumors that display an increased expression of cyclin D1. However, because cyclin D1 exerts its major effects through the Rb protein, such agents would probably not be effective in tumors that lack expression of functional Rb protein. Thus, when agents are designed that target specific cell cycle control proteins, it may be necessary to profile individual tumors for their specific genetic defects and thereby tailor the therapy based on these findings.

Some of the mutations listed in Table I could also increase the resistance of cancer cells to some of the currently used drugs that inhibit DNA synthesis. For example, loss of the Rb gene or enhanced cyclin D1/CDK4 kinase activity could increase the activity of the transcription factor E2F and thereby increase the cellular levels of dihydrofolate reductase, therefore conferring increased resistance to methotrexate. The clinical significance of this possibility remains to be determined.

Because the overexpression of cyclin D1 can enhance the process of gene amplification and thereby contribute to genomic instability, inhibitors of cyclin D1 or cyclin D1/CDK4 activity may be useful in blocking the process of tumor progression and the emergence of drug-resistant variants. Mutations in the p53 and Rb genes can also enhance genomic instability. Further studies are required to determine to what extent some of the other abnormalities listed in Table I might contribute to genomic instability and drug resistance, and whether some of these other defects are also potential targets for cancer therapy.

Thus, within just a few years, studies on cyclins and related genes have revealed new insights into the molecular genetics of cancer, insights which can

hopefully be used to develop new strategies for reducing cancer incidence and mortality.

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See Also the Following Articles

CELL CYCLE CONTROL; P53 TUMOR SUPPRESSOR GENE: STRUCTURE AND FUNCTION; RETINOBLASTOMA TUMOR SUPPRESSOR GENE.

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The Scientific Bases of Cancer Chemoprevention.
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Disturbances in cell cycle control as targets for cancer chemoprevention: the cyclin D1 paradigm

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Abstract. A characteristic feature of the carcinogenic process is the long-latency period. In humans this time interval is usually decades. At the mechanistic level the basis for the latency period most likely reflects the time required for the successive acquisition of numerous mutations in DNA, repetitive cycles of clonal expansion and cell selection, and epigenetic changes in gene expression, in the evolving tumor.

An optimistic aspect of the latency period and the multistage nature of the carcinogenic process is that it offers numerous opportunities for intervention before fully malignant tumors develop, using various approaches including chemoprevention. At the same time, a rationale scientific approach to cancer chemoprevention and the development of more clinically effective agents requires a better understanding of the cellular and molecular events involved in the various stages of the carcinogenic process. A large number of so-called oncogenes and tumor-suppressor genes have been implicated in the multistage carcinogenic process. Functionally, they can be divided into two categories: 1) those that control intracellular circuitry and signal transduction pathways which ultimately influence gene expression, and 2) those that play a direct role in cell surface and extracellular functions.

Recent studies indicate that the function of genes that control the cell cycle, particularly cyclin D1, are also disturbed during the carcinogenic process and that these changes perturb both cell proliferation and genomic instability, thus enhancing the process of tumor progression. Therefore, cyclin D1 and related proteins might be useful biomarkers and also targets for cancer chemoprevention.

Key words: cancer, cell cycle, chemoprevention, cyclins, cyclin-dependent kinases.

Introduction

Chemoprevention is rapidly evolving as a new strategy for cancer control based on pharmacological or nutritional interventions to prevent, block or reverse the process of tumor formation before fully malignant tumors develop.

Carcinogenesis is a multistep process in which several events are required to produce cancer. An understanding of these different steps at the cellular and molecular level is required for a rationale scientific approach to cancer chemoprevention and the development of more clinically effective agents.

Several models have been proposed to explain the process of tumor development.

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Table 1. Cellular and molecular events in cancer development

1. Successive acquisition of numerous mutations in various cellular genes.
2. Repetitive cycles of clonal expansion and cell selection.
3. Epigenetic changes in gene expression.
4. Progressive acquisition of genome instability.

One of these is the model of clonal evolution, first proposed by Nowell in 1976 [1]. In this model, the accumulation of multiple mutations in the progeny of cells derived from a single cell, which originally acquires a cancer predisposing mutation, is responsible for the development of a fully malignant tumor. Subsequent research has elucidated that several types of events are required for this process (Table 1). Thus, the occurrence of multiple mutations is associated with repetitive cycles of cell selection and clonal expansion. Genomic instability plays an important role by facilitating the formation of mutant cells in the expanding tumor population. Mutations which confer a selective growth advantage over the surrounding cells will be retained and will contribute to the formation of sublines that are increasingly abnormal and genetically unstable. Epigenetic (i.e., nonmutational) changes probably also contribute to this process by altering gene expression, although this aspect requires more intensive studies.

The complexity of these events is responsible for a characteristic feature of the carcinogenic process, observed both in experimental models and in humans, which is the long period of latency between the first exposure to carcinogenic factors and the development of a fully malignant tumor. Several types of evidence confirm the existence of the latent period (Table 2) and different factors may affect, both positively and negatively, its length (Table 3).

An optimistic aspect of the latency period is that it offers numerous opportunities for intervention before fully malignant tumors develop. Chemoprevention strategies are possible at different levels. Thus, chemopreventive agents include substances that reduce absorption of carcinogens, such as dietary fibers; substances that reduce the synthesis of carcinogens in the body, such as vitamin C which inhibits the formation of nitrosamines in the stomach; chemicals that inhibit the metabolic activation of carcinogens or enhance their detoxification and/or excretion such as benzyl isothiocyanate, which is present in cruciferous vegetables, selenium, β-carotene and other antioxidants; chemicals that trap ultimate carcinogens preventing their

Table 2. Evidence for the latent period in cancer development

1. Experimental studies.
2. In humans - can be years or decades:
a. radiation exposure;
b. cigarette smoking;
c. occupational carcinogenesis; and
d. chemotherapy.
3. Can be "transplacental".

Table 3. Factors that influence the latent period

1. Carcinogenic dose.
2. Tissue site.
3. Presence of tumor promoters or other factors.
4. Anticarcinogens.

interaction with DNA, such as elagic acid or flavonoids, present in fruits and vegetables. The latter category of compounds is referred to as carcinogen-blocking agents. Other compounds, referred to as promotion-suppressing agents, prevent the evolution of the carcinogenic process in initiated cells. They include retinoids, β-carotene and α-tocopherol, which are present in fruit and vegetables, and other chemicals such as aspirin and other nonsteroidal anti-inflammatory drugs. Experimental and epidemiological studies suggest that other substances, such as organosulfur compounds in garlic and onion, curcumin in tumeric/curry, polyphenols in green tea and various protease inhibitors might also be useful in preventing tumor formation. However, their mechanisms of action are still unknown.

Within the past few decades great advances have been made in understanding the genetic and molecular mechanisms which underlie the process of tumor development. As mentioned above, the major feature of this process is the progressive acquisition of mutations in a variety of cellular genes which eventually leads to the appearance of a fully malignant phenotype. The identification of these genes and the specific mechanisms by which they contribute to this process is essential for developing effective chemopreventive agents. In fact, alterations in these genes might help to identify individuals at risk to develop cancer, provide useful targets for chemoprevention, or provide biological markers to be used as intermediate end points for assessing the efficacy of chemoprevention agents in clinical trials.

The genes involved in the carcinogenic process have been classically subdivided into two major groups [2,3]: 1) oncogenes, which are dominant-acting genes that normally mediate agonist-induced signal transduction pathways and whose increased activity contributes to tumor development; and 2) antioncogenes (or tumor-suppressor genes), which are recessive-acting genes whose functional loss contributes to the progression of cells towards the neoplastic state.

This classification, however, does not indicate the biochemical function of these genes, nor at what level they act to contribute to cancer formation. Therefore, we suggest a new classification scheme for these genes (Table 4). According to this

Table 4. Categories of genes involved in carcinogenesis

1. Intracellular circuitry.
   a. agonist-induced signal transduction;
   b. cell-cycle control and apoptosis;
   c. DNA replication and repair; and
   d. differentiation.
2. Cell surface/extracellular functions:
   adhesion, proteases, angiogenesis, etc.

scheme, the genes mutated in cancer cells can be functionally divided into two major categories: 1) those that control intracellular regulatory circuitry, and 2) those that influence cell surface and extracellular functions. The latter category refers to genes involved in the interactions between tumor cells and the surrounding host environment. They are especially relevant to tumor cell invasion and metastasis and include genes encoding various cell surface proteins, cell adhesion molecules, extracellular matrix proteases and other proteins, and angiogenesis factors.

The first category can be further divided into four subcategories. The genes in the first subcategory are involved in the responses of cells to external regulatory signals. These genes encode growth factors, membrane receptors and all of the coupling proteins which transduce the message through the cytosol to the nucleus to activate or inactivate the transcription of specific genes. Most of the classical oncogenes fit into this subcategory. The second subcategory includes genes involved in the regulation of cell proliferation and programmed cell death (or apoptosis). This category is rapidly expanding due to the increased understanding of the molecular mechanisms regulating the cell cycle machinery. The two major tumor-suppressor genes, Rb and p53, fit into this subcategory. We will discuss this category in greater detail later. The third subcategory includes genes involved in DNA replication, repair and recombination. Mutations in these genes (for example in DNA mismatch repair genes) can lead to genomic instability. Finally, the fourth subcategory refers to genes, such as MyoD, which normally regulate cell differentiation but, if altered, might also contribute to tumor development.

This classification clearly suggests how multiple pathways involved in cell proliferation, cell differentiation and cell death can all contribute to the multistep process of tumor formation, through complex and intricate interactions, many of which are still unknown. From this point of view, the neoplastic phenotype might be considered as a condition in which the normal balance between these diverse cellular processes is altered so as to impair cellular differentiation and enhance cell proliferation.

Cell cycle control and cancer

During the past decade rapid advances have been made in our understanding of the normal cell cycle machinery and there is accumulating evidence that disruption of the normal cell cycle is one of the most important alterations involved in cancer development (for review see [4,5]).

A central role in the control of the eukaryotic cell cycle is played by a family of cyclin-dependent kinases (CDKs) which are regulated by positive regulatory subunits called cyclins. Cyclins control the timing of activation and the substrate specificity of a series of CDKs, which are sequentially activated during specific phases of the cell cycle.

G1 cyclins regulate the progression of cells through the G1 phase and drive entry into the S-phase. Three D-type cyclins, D1, D2 and D3, act at mid-G1 by complexing with either CDK4 or CDK6. Cyclin E acts in late G1 by complexing with CDK2 [5] (Fig. 1).

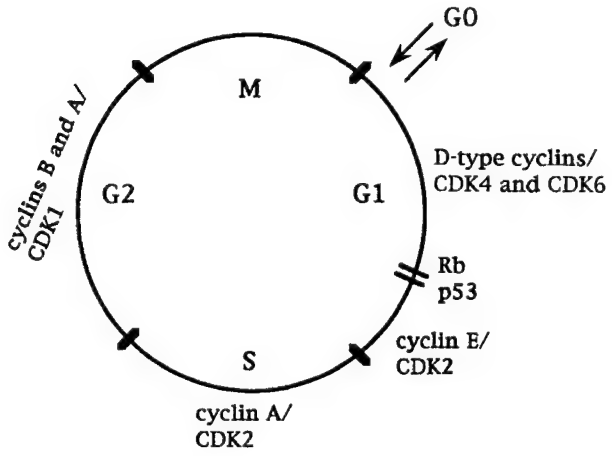

Fig. 1. A schematic diagram of the mammalian cell cycle indicating the GO phase of nondividing cells, the G1 phase when cells enter the cell cycle and prepare for the S-phase, the S-phase in which DNA synthesis occurs, and the G2/M phase in which cells prepare for and undergo mitosis. Also shown are the major cyclins and CDKs which regulate the transitions through these specific phases of the cell cycle; and the Rb and p53 tumor suppressor genes which can act at the G1/S checkpoint (also called restriction point) to inhibit cell cycle progression.

Cyclin accumulation and CDK binding are not the only level of regulation of CDK activity. Both positive and negative phosphorylation events, as well as the association with specific inhibitory proteins also play a critical role in regulating CDK activity. The CDK inhibitors (CDI) identified in mammalian cells are classified into two major categories: 1) p15, p16 and p18 mainly inhibit CDK4 and CDK6 by binding to the CDK subunit itself; and 2) p21, p27, p28 and p57 inhibit a broader range of CDKs by binding to several cyclin/CDK complexes [6,7]. A schematic representation of the multiple mechanisms regulating the activity of the cyclin D1/CDK4 complex is shown in Fig. 2. The same scheme also shows one of the major targets of this complex, the Rb protein, a product of the Rb tumor-suppressor gene. Rb is an inhibitor of cell proliferation and mainly acts in early mid-G1 by binding to E2F, a transcription factor whose activity is required for the G1 to S transition. E2F cannot activate S-phase genes when it is bound to Rb. The binding of Rb to E2F can be prevented by phosphorylation of Rb, which occurs in midlate G1. The cyclin D1/CDK4 complex plays the major role in phosphorylating the Rb protein, thus inhibiting its function: when Rb is phosphorylated, free E2F is released, which then activates the transcription of genes required for progression through the S-phase.

Since the major regulatory events leading to mammalian cell proliferation and differentiation occur in the G1 phase of the cell cycle, deregulated expression of the G1 cyclins and CDKs might cause a loss of cell cycle control and thus enhance oncogenesis.

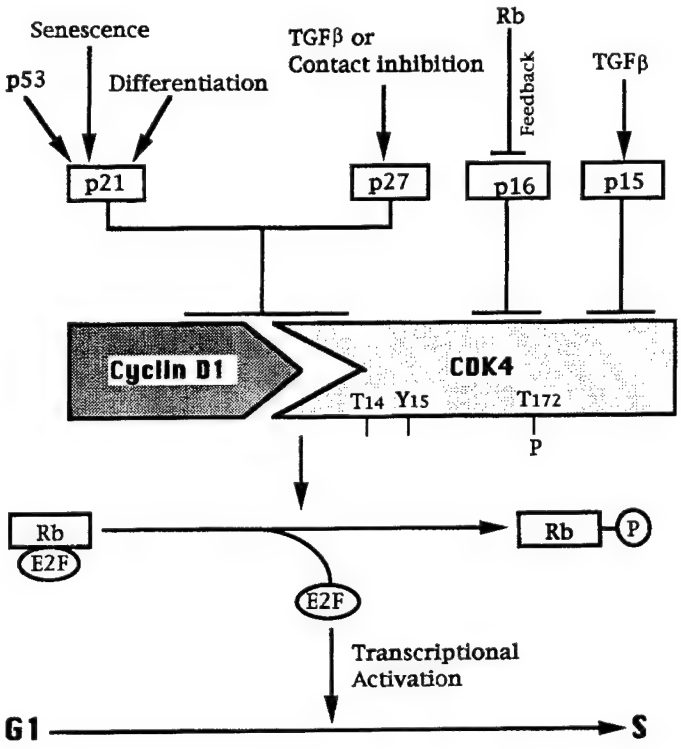

Fig. 2. Schematic representation of the multiple levels of regulation of cyclin/CDK activity. The cyclin D1/CDK4 complex is shown but similar mechanisms also regulate the activity of other cyclin/CDK complexes. The binding of cyclin D1 to CDK4 is essential to activate the kinase activity. However, phosphorylation of specific sites (tyrosine 172) and dephosphorylation of others (tyrosine 15 and threonine 14) is also necessary for kinase activity. Specific kinases and phosphatase regulate the phosphorylation and dephosphorylation of these sites during the cell cycle. Finally, a group of inhibitory proteins, which are in turn regulated by several external factors, also contribute to regulate the final activity of cyclin/CDK complexes. Also shown is the product of the Rb tumor suppressor gene, a major target of cyclinD1/CDK4 complex, which can inhibit cell cycle progression by binding to and blocking the activity of the E2F transcription factor, whose activity is essential for S-phase progression. Phosphorylation by cyclin D1/CDK4 (and by cyclin E-A/CDK2 complexes) prevents Rb from binding to E2F, thus allowing the G1 to S progression.

Cyclin D1 plays multiple roles in cancer development and is a useful target for cancer chemoprevention

Several types of evidence have been obtained which implicate cyclin D1 in the process of neoplastic transformation. Thus, Rat-6 fibroblasts engineered to stably overexpress cyclin D1 displayed a decrease in the duration of G1, increased growth, decreased cell size; and they induced tumors when injected into nude mice [8]. Furthermore, cyclin D1 was shown to cooperate in vitro with other oncogenes to induce cell transformation [9,10], and overexpression of a MMTV-cyclin D1

construct in mammary cells in transgenic mice was associated with an increase in mammary hyperplasia and mammary carcinomas [11]. Taken together, these results provide direct evidence that overexpression of cyclin D1 can cause disturbances in cell cycle control and enhance tumorigenesis both in vitro and in vivo.

The chromosome 11q13 region is amplified in a variety of human tumors, including esophageal tumors. Indeed, about 20–50% of esophageal carcinomas display amplification of the hst1 and int2 genes which are located on chromosome 11q13. However, little or no expression of these two genes is detectable in the corresponding cells. Thus, when it was found that cyclin D1 mapped to the chromosome 11q13 locus, it was of interest to examine the status of the cyclin D1 gene in esophageal carcinomas. Our laboratory discovered that there was a 3- to 10-fold amplification of the cyclin D1 gene in about 30% of squamous esophageal tumors and several esophageal carcinomas cell lines. We also demonstrated by western blot analysis and immunostaining that cyclin D1 protein expression level was increased in both the cell lines and primary tumors [12].

We further observed that all of the tumors that showed amplification and overexpression of cyclin D1 were positive by immunostaining for Rb expression (group 1). In the remaining tumors, in which cyclin D1 was not overexpressed, some had undetectable Rb protein expression (group 2) but the remainders were Rb-positive (group 3), although we did not exclude the possibility that in some of the Rb-positive cases a mutant protein was expressed [13]. This reciprocal relationship between overexpression of cyclin D1 and loss of expression of the Rb protein was statistically significant and suggested a model in which, during the multistep evolution of esophageal tumors, the normal inhibitory role of the Rb protein was abrogated by various mechanisms including: 1) loss of expression of the Rb protein (group 2); and 2) increased expression of cyclin D1 (group 1); or other, at that time unknown, mechanisms (group 3).

This model has been subsequently confirmed and extended to other types of tumors. Alternative mechanisms for Rb inactivation later became apparent in the group 3 tumors. These include loss of inhibitory proteins (such as p16) or amplification and overexpression of CDK4, which have the same final effects on this important pathway of cell-growth regulation.

This example provides a paradigm which demonstrates that the analysis of entire pathways of cell-growth regulation is more informative than simply the study of single genes in understanding tumor cell biology. In fact, tumors that are heterogeneous with respect to mutations of specific genes might actually display deregulation in a common regulatory pathway, and thus have similar biological properties.

Cyclin D1 has also been found to be amplified and/or overexpressed in a variety of human tumors, including breast cancers [14]. As mentioned above, to develop more effective strategies for cancer prevention and treatment we need a better understanding of all of the stages of the carcinogenic process, including the initiating events. These events cannot be readily studied in humans. An experimental model is needed that mimics the human disease and allows one to elucidate the first phases of

the tumorigenic process, and whether it can be manipulated by treatment of the host. Thus, it was of interest to determine whether carcinogen-induced rat mammary tumors, which represent a widely used experimental model for mammary tumorigenesis, also display abnormalities in the expression of cyclin D1. Indeed, we found that primary rat mammary carcinomas induced by the carcinogen N-methyl-N-nitrosourea (NMU) frequently show deregulated expression of cyclin D1 and other cell cycle-related genes [15], thus confirming the utility of this model for studies on mammary tumorigenesis.

Since cyclin D1 is part of a very large amplicon, which includes several genes; its amplification and overexpression in tumor cells does not exclude the possibility that genes in this amplicon, other than cyclin D1, play an important role in oncogenesis. To address this question, we introduced an antisense cyclin D1 cDNA construct into a human esophageal cell line which has cyclin D1 amplification and overexpression, and then analyzed possible effects on the growth and tumorigenicity of these cells. We found that the reduction in expression of cyclin D1 in these derivatives was associated with a marked inhibition of both anchorage-dependent and -independent growth in vitro and a complete loss of tumorigenicity when these cells were injected into nude mice [16]. These results provided direct evidence that overexpression of cyclin D1 in these tumor cells plays a critical role in maintaining their abnormal growth and tumorigenicity.

Studies on the cell cycle have shown that normal cells have checkpoint controls at the G1/S and G2/M transition which delay further cell cycle progression to permit repair of damaged DNA. A defect in these cell cycle checkpoints due to loss of the p53 tumor suppressor gene has been associated with genomic instability. Deregulated expression of cyclin D1 can also disrupt normal cell cycle control, and therefore, it might also enhance genomic instability. To test this hypothesis we overexpressed cyclin D1 in a rat liver epithelial cell line which has a normal p53 gene, and using a specific cell selection system, we evaluated the occurrence of gene amplification which is a good marker of genomic instability. We observed that while the occurrence of gene amplification was virtually absent in the parental cell line, the cyclin D1 overexpressing derivatives showed a very high frequency of gene amplification [17]. This finding suggests that overexpression of cyclin D1 can disrupt genomic integrity and thereby accelerate the process of tumor progression.

Thus, all of the above studies provide evidence for multiple roles of cyclin D1 overexpression in tumorigenesis, since overexpression of this gene could promote tumor progression by enhancing genomic instability, and also play a role in maintaining the transformed phenotype in certain types of tumor cells.

To extend the relevance of these findings to the development of human tumors in vivo, we have analyzed the expression of cyclin D1 in a variety of human tumors including esophageal, gastric, colon and breast cancers, by using a simple immunostaining procedure. Cyclin D1 overexpression was observed in all of these types of tumors, and ranged between 30–60% of the samples analyzed. An interesting finding was that cyclin D1 overexpression can be an early event in the multistep process of carcinogenesis, since cyclin D1 overexpression was detected in adenomatous polyps

in the colon, in Barrett's esophagus and in situ carcinomas of the breast ([18,19] and unpublished data).

These findings, together with the above-mentioned results, and findings by other investigators, emphasize the importance of increased expression of cyclin D1 in the development of human tumors. They also suggest that cyclin D1 or cyclin D1/CDK4 activity might be useful targets for cancer chemoprevention, since this abnormality is a fairly early event in the process of tumor development and may directly contribute to tumor progression.

Conclusion

In the last few decades, enormous advances have been made in our understanding of cancer causation and formation. There is now compelling evidence that cancer arises via a multistep process and that at a molecular level this process includes the progressive accumulation of genetic changes in a variety of cellular genes. The identification of these genes and the profound insights obtained into the biochemistry and molecular biology of cancer cells have shifted the focus of cancer research to the molecular level. Thus, we have entered the era of "molecular oncology" which should provide a link between basic research and cancer prevention and treatment.

The studies on cyclin D1 provide a paradigm of how advances in the genetics, molecular biology and biochemistry of cancer cells can contribute to the development of new agents for cancer chemoprevention and chemotherapy. The identification of other molecular markers which can alone, or in combination with cyclin D1, contribute to the process of tumor formation is expected to have a major impact on the development of more effective strategies for reducing both cancer incidence and mortality.

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Effects of Cyclin E Overexpression on Cell Growth and Response to Transforming Growth Factor β Depend on Cell Context and p27^{Kip1} Expression[1]

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Abstract

Human tumors often display increased expression of cyclin E, suggesting that this might contribute to their abnormal growth. However, we reported recently that overexpression of a human cyclin E cDNA in the nontransformed mouse mammary epithelial cell line HC11 resulted in increased expression of the cell cycle-inhibitory protein p27^{Kip1} and inhibition of cell growth. To further address the role of cell context, in the present study we analyzed in parallel the effects of cyclin E overexpression in two fibroblast cell lines (Rat1 and NIH3T3) and three nontumorigenic mammary epithelial cell lines (the human cell lines 184B5 and MCF-10F and the mouse cell line HC11). This was associated with increased cyclin E-associated kinase activity in Rat1, NIH3T3, and MCF-10F cells but not in HC11 and 184B5 cells. The derivatives of the latter two cell lines showed increased expression of the p27^{Kip1} protein and inhibition of cell growth. There was a shortening of the G_1 phase in the derivatives of the Rat1 and MCF-10F cells but not in the derivatives of the other three cell lines. Contrary to a previous hypothesis, overexpression of cyclin E was not able to confer anchorage-independent growth in any of these cell lines. However, overexpression of cyclin E was associated with increased resistance to transforming growth factor β-mediated growth inhibition in the 184B5 and HC11 cells and a decrease in transforming growth factor β stimulation of the Rat1 and NIH3T3 fibroblasts. Thus, overexpression of the same cyclin E cDNA has cell type-specific effects on various growth parameters. Therefore, additional studies are required to better understand the significance of the frequent increase of cyclin E expression in human tumors.

Introduction

Cyclins are a family of genes involved in the regulation of cell cycle progression in eukaryotes through mechanisms that have been highly conserved during evolution (1–3). They function essentially by controlling the timing of activation and the substrate specificity of a series of Cdks[4] that are sequentially activated during the cell cycle. Several cyclins and Cdks have been identified in mammalian cells. Specific cyclins bind to specific Cdks, thus activating their kinase activity. Each of these cyclin/Cdk complexes is activated at a specific point during the cell cycle and has a specific set of substrates (1–6).

G1 cyclins regulate the progression of cells through the G_1 phase of the cell cycle and drive entry into S phase. Three D-type cyclins, D1, D2, and D3, act at mid-G_1 by complexing with either Cdk4 or Cdk6 (1, 3, 7). Cyclin E acts in late G_1 by complexing with Cdk2 (8, 9).

Cyclin E is a nuclear protein originally isolated by screening human cDNA libraries for genes that could complement the loss of G1 cyclins in *Saccharomyces cerevisiae* (10, 11). The cyclin E/Cdk2 complex shows strong kinase activity shortly before cells enter S phase and leads to further phosphorylation of the pRb protein (12–14). The accumulation and binding of cyclin E to Cdk2 are not the only mechanisms of regulating cyclin E/Cdk2 kinase activity. Both positive and negative phosphorylation events, as well as association with specific inhibitory proteins, also contribute to this regulation. The activity of the cyclin E/Cdk2 complex is mainly regulated by members of a family of CDIs, which include p21^{Waf1} (also designated Cip1, Pic1, Sd1, and Cap20) and p27^{Kip1} (also called Ick and Pic2; reviewed in Refs. 2 and 15–17).

The accumulation of cyclin E and activation of the cyclin E/Cdk2 complex is a rate-limiting event for the G_1-to-S transition. In fact, antibodies to cyclin E inhibit entry into S phase when injected into cells during the G_1 phase of the cell cycle (18). On the other hand, overexpression of cyclin E accelerates the G_1-to-S transition, decreases cell size, and reduces the serum requirement for growth in human and rat fibroblasts (18–20). Although both cyclins D1 and E play a role in phosphorylating the Rb tumor suppressor protein in mid-late G_1, the functions of these two cyclins are not redundant. In fact, overexpression of cyclin D1, but not cyclin E, is associated with increased phosphorylation of the Rb protein (21).

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[4] The abbreviations used are: Cdk, cyclin-dependent kinase; CDI, cyclin-dependent kinase inhibitory protein; TGF, transforming growth factor; Rb, retinoblastoma.

Moreover, in contrast to the results obtained with antibodies to cyclin D1 (22), injection of anti-cyclin E antibodies arrests cells in G_1, even in Rb minus cells (18). In addition, the cyclin D1/Cdk4 complex can phosphorylate *in vitro* the pRb-related protein p107 but not the pRb-related p130 protein Rb2, whereas the contrary is true for the cyclin E/Cdk2 complex (23, 24).

During the past decade, advances made in our understanding of the cell cycle machinery have clearly indicated that disruption of the normal cell cycle is one of the most important alterations involved in cancer development (2, 3, 16). Because the major regulatory events leading to mammalian cell proliferation and differentiation occur in the G_1 phase of the cell cycle, deregulated expression of the G1 cyclins and Cdks might cause loss of cell cycle control and thus enhance tumorigenesis.

Increased expression of multiple cyclin E-related proteins has been reported in several human malignancies (25, 26). In breast cancer cell lines, the increased expression of cyclin E has been associated with increased cyclin E-associated kinase activity (27). However, we reported recently that overexpression of a human cyclin E cDNA in the nontransformed mouse mammary epithelial cell line HC11 was associated with increased expression of the cell cycle-inhibitory protein $p27^{Kip1}$ and inhibition rather than stimulation of cell growth (28). These findings are in contrast with the above-mentioned results (18–20) with rat and human fibroblasts.

To further address the role of cyclin E in cell growth control and tumorigenesis, in the present study we analyzed, in parallel, the effects of cyclin E overexpression in two fibroblast cell lines and in three nontumorigenic mammary epithelial cell lines. We found that overexpression of the same cyclin E cDNA exerted cell-specific effects on various parameters, including doubling time, saturation density, plating efficiency, cell cycle distribution, cyclin E-associated kinase activity, and the level of expression of $p27^{Kip1}$. We also show that overexpression of cyclin E is not able to confer anchorage-independent growth in any of the cell lines analyzed, but it does modulate the responses of some of these cell lines to TGF-β. The implications of these findings are discussed.

Results

Generation of Derivatives That Stably Overexpress Cyclin E. To further address the role of cyclin E in cell cycle control and transformation, we analyzed the phenotypes of derivatives of four different cell lines that stably overexpress cyclin E and also compared the results obtained with derivatives of the HC11 mouse mammary epithelial cell lines, the properties of which we have described previously (28). The four new cell lines were Rat1 fibroblasts, NIH3T3 mouse fibroblasts, and 184B5 and MCF-10F nontransformed human mammary epithelial cells. These cell lines were transduced with the same human cyclin E cDNA expressed from a retroviral promoter used in our previous studies with the HC11 cell line (28). After selection for resistance to hygromycin (*hph*), pools of thousands of resistant colonies were obtained, both from the cultures infected with the PMV12-cycE construct and the cultures infected with the PMV12pl vector (vector control cells), and used for the studies described below.

Expression of the exogenous cyclin E gene was verified by Western blot analysis using either a polyclonal anti-cyclin E antibody or an antihuman cyclin E monoclonal antibody that specifically recognizes human cyclin E. All of the above-mentioned five cell lines used in the present studies expressed variable levels of the major endogenous cyclin E band, which was about M_r 55,000 in the rat and mouse cells and about M_r 52,000 in the human cells (Figs. 1 and 2; Refs. 18, 19, and 28). As reported previously (28), the polyclonal antibody mainly detected a M_r 50,000 band corresponding to the exogenous cyclin E in the overexpressor cells (Fig. 6 and data not shown), whereas the monoclonal antibody also detected a prominent exogenous M_r 42,000 band (Ref. 28; Figs. 1 and 2). As shown in Figs. 1 and 2, we used the monoclonal antibody to rule out the possibility that the different effects seen in the different cell lines might have been because of different levels of expression of the exogenous M_r 42,000 protein in the various cell lines. The polyclonal anti-cyclin E antibody, which only recognizes the M_r 50,000 exogenous cyclin E band, was used (see Fig. 6) because it enabled us to detect both the exogenous and the endogenous cyclin E proteins. We have previously reported the overexpression of cyclin E in HC11 and Rat1 cells (28).

Overexpression of the exogenous cyclin E cDNA was verified by Northern blot analysis, and nuclear localization of the cyclin E protein and its increased level in the derivatives of the five cell lines were confirmed by immunostaining with an anti-cyclin E antibody (data not shown).

It is of interest that in multiple transduction experiments with the 184B5 cells the number of hph^+ clones obtained after transduction with the PMV12-cycE construct was lower than the number obtained after transduction with the control PMV12pl construct. Furthermore, with the 184A1 nontumorigenic human mammary epithelial cell line (29), we were unable to obtain any hph^+ colonies that expressed the exogenous cyclin E (data not shown). These findings suggest that overexpression of this cyclin E cDNA was somewhat toxic to the 184B5 cell line and highly toxic to the 184A1 cell line. Moreover, the cyclin E overexpressing pools of the 184B5 cells showed only a moderate level of expression of the exogenous cyclin E bands, because the level was considerably lower than that obtained with the MCF-10F and Rat1 cell lines (Fig. 2). We also tried to obtain single clones of 184B5 cells that overexpressed the exogenous cyclin E. However, when we analyzed several hph^+-resistant clones for expression of cyclin E, we found that they expressed only the M_r 42,000 exogenous cyclin E (Fig. 2, last two lanes), which might correspond to a spliced form of cyclin E reported previously to be unable to activate Cdk2 (30). These findings are consistent with studies described below indicating that ectopic expression of this cyclin E cDNA in 184B5 cells inhibits their growth, thus resembling the situation with HC11 cells (28).

Effects of Cyclin E Overexpression on Cell Cycle Kinetics and Cell Growth. To evaluate the phenotypic effects of cyclin E overexpression, several parameters were examined, in parallel, in the vector control pools of the three cell

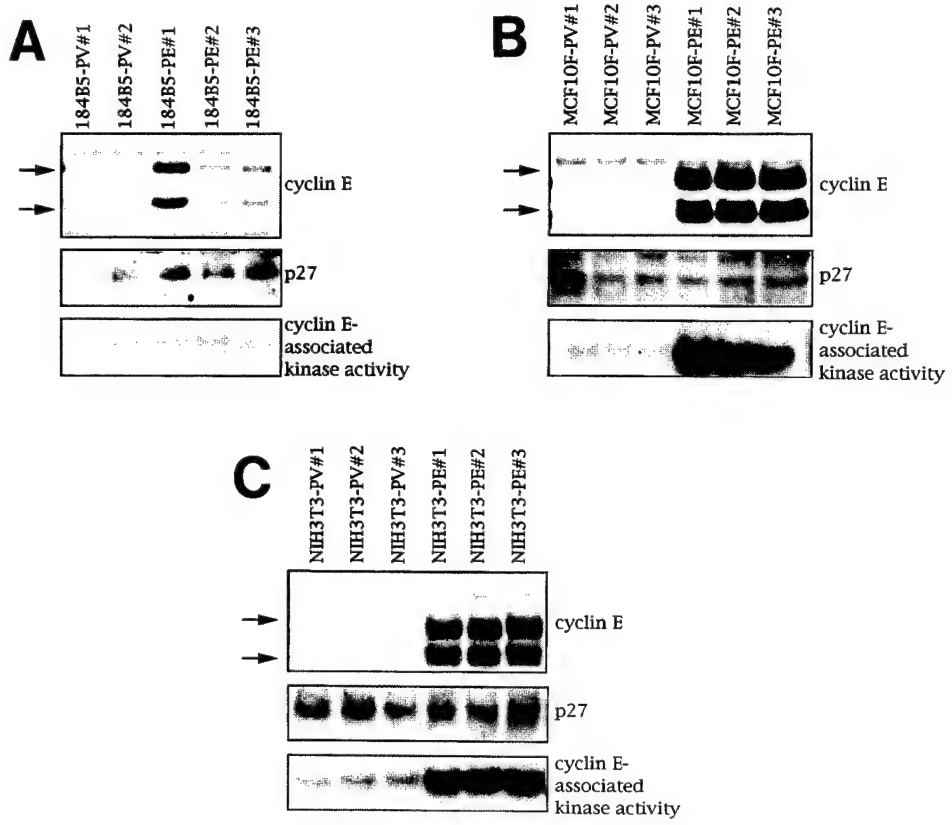

Fig. 1. Effects of stable overexpression of cyclin E in the 184B5 (*A*) and MCF-10F (*B*) human nontumorigenic mammary epithelial cell lines and in NIH3T3 (*C*) mouse fibroblasts. Exponentially growing cultures of vector control (*PV*) and cyclin E-overexpressing pools (*PE*) of each cell line were analyzed. For Western blot analyses, 50 μg of proteins in whole-cell lysates were resolved by 10% SDS-PAGE and transferred to an Immobilon membrane. Duplicate blots were probed with the anti-cyclin E (*top panels*) and the anti-$p27^{Kip1}$ (*middle panels*) antibodies, and immunoreactive bands were detected by enhanced chemiluminescence. The monoclonal antihuman cyclin E antibody was used for detecting cyclin E, because it recognizes both the M_r 50,000 and the M_r 42,000 bands corresponding to the exogenous cyclin E. *Arrows*, positions of the exogenous cyclin E bands. For the histone H1 kinase activity (*bottom panels*), 50 μg of whole-cell lysates were used for derivatives of the MCF-10F and NIH3T3 cells, and 100 μg were used for derivatives of the 184B5 cells. For additional details, see "Materials and Methods."

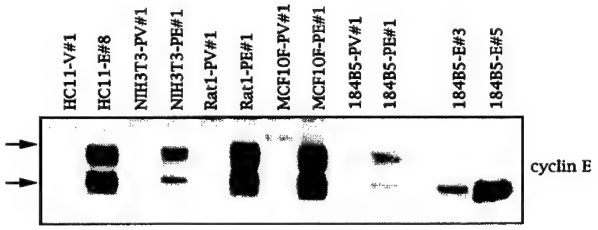

Fig. 2. Western blot analysis for human cyclin E proteins in various cell lines are shown. *Lanes 1* and *2* are previously described (28) vector control and cyclin E-overexpressor derivatives of the HC11 mouse mammary cell line, respectively. Also shown are vector control (*PV*) and representative cyclin E-overexpressing pools (*PE*) of NIH3T3, Rat1, MCF-10F, and 184B5 cells. The monoclonal antihuman cyclin E antibody was used for detecting cyclin E. *Arrows*, positions of the M_r 50,000 and M_r 42,000 bands corresponding to the exogenous cyclin E. The *last two lanes* are two single cyclin E-overexpressing clones (*E#3* and *E#5*) of the 184B5 cell line that express only the M_r 42,000 exogenous cyclin E protein.

lines, NIH3T3-PV, 184B5-PV, and MCF10F-PV, and in the corresponding cyclin E overexpressor pools, NIH3T3-PE, 184B5-PE, and MCF10F-PE (Table 1). The effects of cyclin E overexpression on cell cycle kinetics and cell growth have been reported previously in Rat1 cells (19, 28) and HC11 cells (28).

Overexpression of cyclin E in NIH3T3 cells had no effect on the distribution of cells in the cell cycle. Growth curves of monolayer cultures also indicated no appreciable differences in the doubling times or saturation densities between cyclin E-overexpressor derivatives of NIH3T3 cells and the corresponding vector control cells. However, the NIH3T3-PE cells showed a higher plating efficiency compared with the NIH3T3-PV cells (25% *versus* 14%; Table 1). These effects were associated with an increase in cyclin E-associated kinase activity, and no changes were observed in the cellular level of the $p27^{Kip1}$ protein (Fig. 1*C*).

The cyclin E-overexpressing derivatives of MCF-10F cells displayed a reduction in the percentage of cells in the G_1 phase (about 52% *versus* 62%) and an increase in the percentage of cells in S phase (about 15% *versus* 8%), when compared with the vector control cells (Table 1). In addition, the MCF10F-PE cells displayed a slightly shorter doubling time than the vector control cells and an increased saturation density (Table 1). Unexpectedly, the plating efficiency of MCF10F-PE cells was reduced when compared to MCF10F-PV cells (4.0% *versus* 6.6%). The cyclin E-associated kinase activity was markedly increased in the MCF10F-PE cells, and no change was observed in the level of the $p27^{Kip1}$ protein in these cells (Fig. 1*B*).

The cyclin E-overexpressing pools of the 184B5 cell line showed an increase in the percentage of cells in the G_1 phase of the cell cycle (about 65% *versus* 55%) and a reduction of cells in S phase (about 23% *versus* 30%) when compared with the corresponding vector control cells. These derivatives of 184B5 cells also displayed a longer doubling time, a lower saturation density, and decreased plating efficiency when compared with the vector control cells (Table 1).

Table 1 Effects of cyclin E overexpression on cell cycle distribution and growth properties in different cell lines

All assays were performed in triplicate, and all experiments were repeated at least twice. The data reported are the results of a typical experiment for a representative cell line. Individual points are the means of triplicate determinations. SD for individual points were less than 25% of the mean. Similar results were obtained in replicate experiments.

Cell line	D.T.[a] (h)	S.D.[b] ($\times 10^6$)	P.E.[c] (%)	G_0-G_1	S	G_2-M	A.I.G.[d] (%)	$p27^{Kip1}$[e]	Cyclin E kinase activity[e]
					(%)[f]				
Rat1-PV#1	17.0	4.0	14.0	58.8	30.8	10.3	0		
Rat1-PE#1	17.0	5.2	29.0	35.9	46.9	17.2	0	No change	Increase
MCF10F-PV#1	30.0	3.5	6.6	61.9	8.5	29.7	0		
MCF10F-PE#1	27.8	4.3	4.0	52.3	15.1	32.7	0	No change	Increase
NIH3T3-PV#1	15.0	2.7	14.0	55.1	34.3	10.6	0		
NIH3T3-PE#1	15.0	2.6	25.0	54.9	34.9	10.2	0	No change	Increase
184B5-PV#1	25.8	2.6	11.6	55.1	29.7	15.2	0		
184B5-PE#1	29.8	1.5	6.4	68.2	22.6	9.1	0		
184B5-PE#2	28.0	1.7	8.0	62.7	26.7	10.6	0	Increase	No change
HC11-V#1[g]	14.6	11.4	8.0	28.9	53.7	17.4	1.7		
HC11-E#8	18.0	6.6	4.2	43.5	45.9	10.6	0.1	Increase	No change

[a] D.T., doubling time, corresponds to the initial exponential phase of the cell growth.
[b] S.D., saturation density, represents the total number of cells per 35-mm well when the cultures reached a plateau.
[c] P.E., plating efficiency.
[d] A.I.G., anchorage-independent growth, expressed as colony-forming efficiency in soft agar.
[e] These results refer to the situation in the cyclin E-overexpressing derivatives of each cell line when compared to the corresponding vector control cells.
[f] Exponentially growing cultures of the indicated cell lines were analyzed by flow cytometry. The values represent the percentage of the total cell population in each phase of the cell cycle.
[g] Most of the data regarding the derivatives of HC11 cells have been reported previously (Cancer Res., 56 1389–1399, 1996). For additional details, see "Materials and Methods."

These differences, although not dramatic, were reproducible in multiple experiments and were observed in separately isolated pools of the cyclin E-overexpressing 184B5 cells (data not shown). Moreover, these changes were related to the level of expression of the exogenous cyclin E (compare the data on the 184B5-PE#1 and 184B5-PE#2 pools in Fig. 1*A* and Table 1). As reported previously for the mouse mammary epithelial cell line HC11 (28), when exponentially growing cells were analyzed for the amount of cyclin E-associated kinase activity, no significant increase was observed between the cyclin E-overexpressing 184B5 cells and the vector control cells. Exponentially dividing cultures of the cyclin E-overexpressing pools also displayed increased expression of the $p27^{Kip1}$ protein when compared with the control pools (Fig. 1*A*). As mentioned above, single clones of 184B5 cells isolated from cultures transduced with the cyclin E vector expressed only the M_r 42,000 cyclin E protein band (Fig. 2, last two lanes) and did not display an increase in cyclin E-associated kinase activity (data not shown).

Overexpression of Cyclin E Is Not Sufficient to Induce Anchorage-independent Growth. A recent report suggested that lack of activation of cyclin E-associated kinase activity is responsible for the G_1 block observed when untransformed human diploid fibroblasts are maintained in suspension rather than allowed to become adherent to a growth surface (31). This conclusion was based on the finding that activation of cyclin E/Cdk2, which is required for the G_1-to-S transition, was seen in late G_1 in adherent fibroblasts but not in fibroblasts maintained in suspension. On the other hand, in anchorage-independent transformed fibroblasts, the cyclin E/Cdk2 complex was activated in late G_1, even when the cells were grown in suspension. These investigators also reported that the lack of cyclin E/Cdk2 activity in the suspended untransformed fibroblasts was associated with increased expression of $p27^{Kip1}$ (31).

It was of interest, therefore, to examine the responses of our derivatives of Rat1 and NIH3T3 fibroblasts that stably overexpress cyclin E to growth in suspension, using a protocol reported previously (32). Asynchronous adherent cultures of Rat1-PV, Rat1-PE, NIH3T3-PV, and NIH3T3-PE cells were trypsinized and replated in parallel onto nontreated tissue culture plastic dishes (adherent cultures) and onto agar-coated dishes (nonadherent cultures), as described in "Material and Methods." After 48 h, cells from both types of cultures were collected, and their cell cycle distribution was analyzed by flow cytometry. Representative results are shown in Fig. 3. As expected, with the adherent cultures of all four cell lines, which were growing exponentially, cells were distributed throughout the cell cycle (Fig. 3*B*). About 55% of the NIH3T3-PV and NIH3T3-PE cells were in G_1. The respective values for Rat1-PV and Rat1-PE were 59 and 36%, respectively.

The suspension cultures gave very different results (Fig. 3*A*). About 85% of the NIH3T3-PV, NIH3T3-PE, and Rat1-PV cells arrested with a 2N DNA content after having been grown in suspension for 48 h, indicating cell cycle arrest at some point in G_0 or G_1. On the other hand, when the Rat1-PE cells were grown in suspension for 48 h the distribution of cells in the G_0-G_1, S and G_2-M phases of the cells was 53.4, 37.1, and 9.5%, respectively. Therefore, after growth in suspension for 48 h, the Rat1 cyclin E-overexpressing cells had not undergone significant growth arrest. However, the Rat1-PE cells did show a progressive accumulation of cells in G_0-G_1 at later time points; thus, after 96 h in suspension, about 80% of the cells were in G_0-G_1 (data not shown).

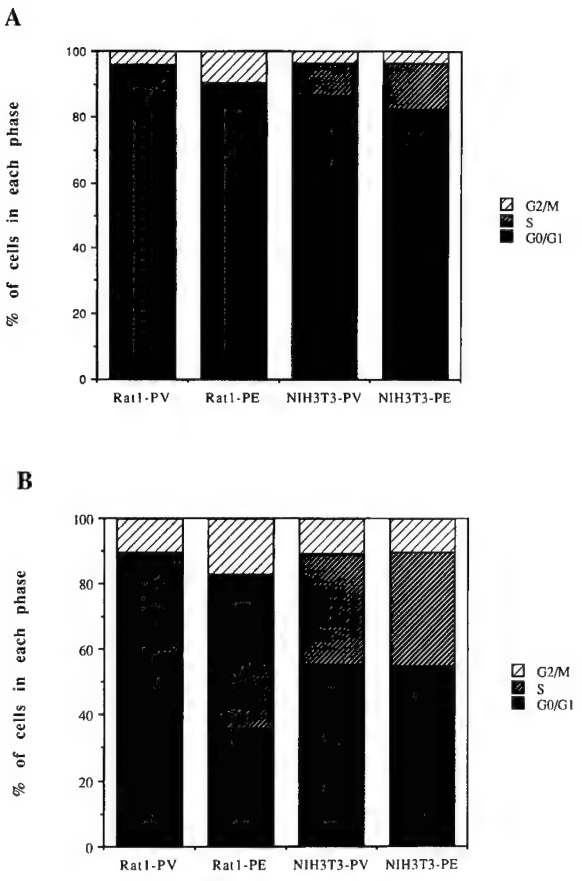

Fig. 3. Accumulation of cells in G_0-G_1 in the absence of cell adherence. In *A*, asynchronous adherent cultures of the indicated vector control (*PV*) and cyclin E-overexpressing pools (*PE*) were trypsinized and replated onto agar-coated dishes. After 48 h, the suspended cells were collected, and their cell cycle distribution was analyzed by flow cytometry. In *B*, the cell cycle distribution of parallel adherent cultures of the same cell lines is shown for comparison. Cells remained >90% viable after 2 days in suspension.

To extend this analysis, we analyzed synchronized cultures. Adherent monolayer cultures of Rat1-PV and Rat1-PE cells were arrested in G_0 for 72 h by serum starvation, trypsinized, and then replated into untreated and agar-coated tissue culture dishes. After serum re-stimulation, the cell cycle distribution of both cultures was analyzed by flow cytometry at various times thereafter. As shown in Fig. 4, adherent cultures of both Rat1-PV and Rat1-PE were in S phase after about 14 h, and thereafter, proceeded through G_2-M and reentered G_1. On the other hand, the progression from G_0 to S was markedly reduced in nonadherent cultures of both Rat1-PV and Rat1-PE cells (Fig. 4). It is of interest that whereas most of the Rat1-PV cells were cell cycle arrested after 24 h of growth in suspension, *i.e.*, >85% of these cells were in G_1, with Rat1-PE cells, 69% of the cells were in G_1 and 22% were in S phase after 24 h. However, when kept in suspension for 72 h, >85% of the Rat1-PE cells accumulated in G_0-G_1 (data not shown).

We also examined the ability of the above described cell lines to undergo cell division in suspension culture by doing serial cell counts for up to 48 h after the cells were transferred to agar-coated plates (Fig. 5). It was apparent that the NIH3T3-PV, NIH3T3-PE, and Rat1-PV cells did not show a significant increase in cell number. However, the Rat1-PE cultures underwent about one doubling of the cell population within the first 24 h but did not increase in number thereafter. These data, together with the cell cycle data in Fig. 4, indicate that overexpression of cyclin E in NIH3T3 cells does not confer the ability to grow in suspension. Overexpression of cyclin E in Rat1 cells does allow them to undergo about one cell division but does not allow continuous cell replication in suspension cultures.

To extend these results, we tested whether the cyclin E-overexpressing derivatives of the various cell lines used in this study had an enhanced ability to form colonies in soft agar when compared with the vector control cells. However, neither the vector control or the cyclin E derivatives of the Rat1, NIH3T3, MCF-10F, or 184B5 cells were able to form colonies in soft agar, even in medium with a high concentration of serum (up to 30%; Table 1 and data not shown). We reported previously that HC11 cells are able to grow in soft agar, although with a very low efficiency, and this ability was actually reduced in the cyclin E-overexpressor derivatives (28). Therefore, overexpression of cyclin E does not confer anchorage-independent growth to any of the five cell lines listed in Table 1.

It was also of interest to examine cyclin E-associated kinase activity in extracts obtained from adherent and nonadherent cultures of Rat1-PV and Rat1-PE cells. Adherent exponentially growing cultures of Rat1-PE cells showed about a 5-fold higher cyclin E-associated kinase activity than the comparable vector control cells. However, after growth in suspension for 48 h, both cell lines showed a marked decrease in cyclin E-associated kinase activity, although this activity remained slightly higher in the cyclin E-overexpressing cells (Fig. 6*A*). The decrease in cyclin E-associated kinase activity was not associated with a decrease in the level of the cyclin E protein, but as reported previously for KD and IMR90 cells (31), growth in suspension led to a marked increase in the level of expression of the $p27^{Kip1}$ protein (data not shown).

We also analyzed cyclin E expression and cyclin E-associated kinase activity in adherent and nonadherent cultures of G_0-synchronized Rat1-PV and Rat1-PE cells. Serum-starved cells were restimulated with serum (as described in Fig. 4), and protein extracts were prepared from cells collected at various times after the addition of serum. In adherent cultures, the level of expression of the endogenous cyclin E protein in both Rat1-PV and Rat1-PE cells peaked at about 14 h, corresponding to the G_1-S boundary, and was decreased at 20 h (Fig. 6*B*). In nonadherent cultures of both cell lines, the level of expression of the endogenous cyclin E was increased at 14 h but remained high at 20 h. The latter finding suggested that the nonadherent cells arrested in late G_1, after the induction of cyclin E. As expected, throughout these studies, there was constitutive expression of the exogenous M_r 50,000 cyclin E protein in the Rat1-PE cells (Fig. 6*B*). In the above studies, we were surprised to observe an appreciable amount of the endogenous cyclin E protein in the cells

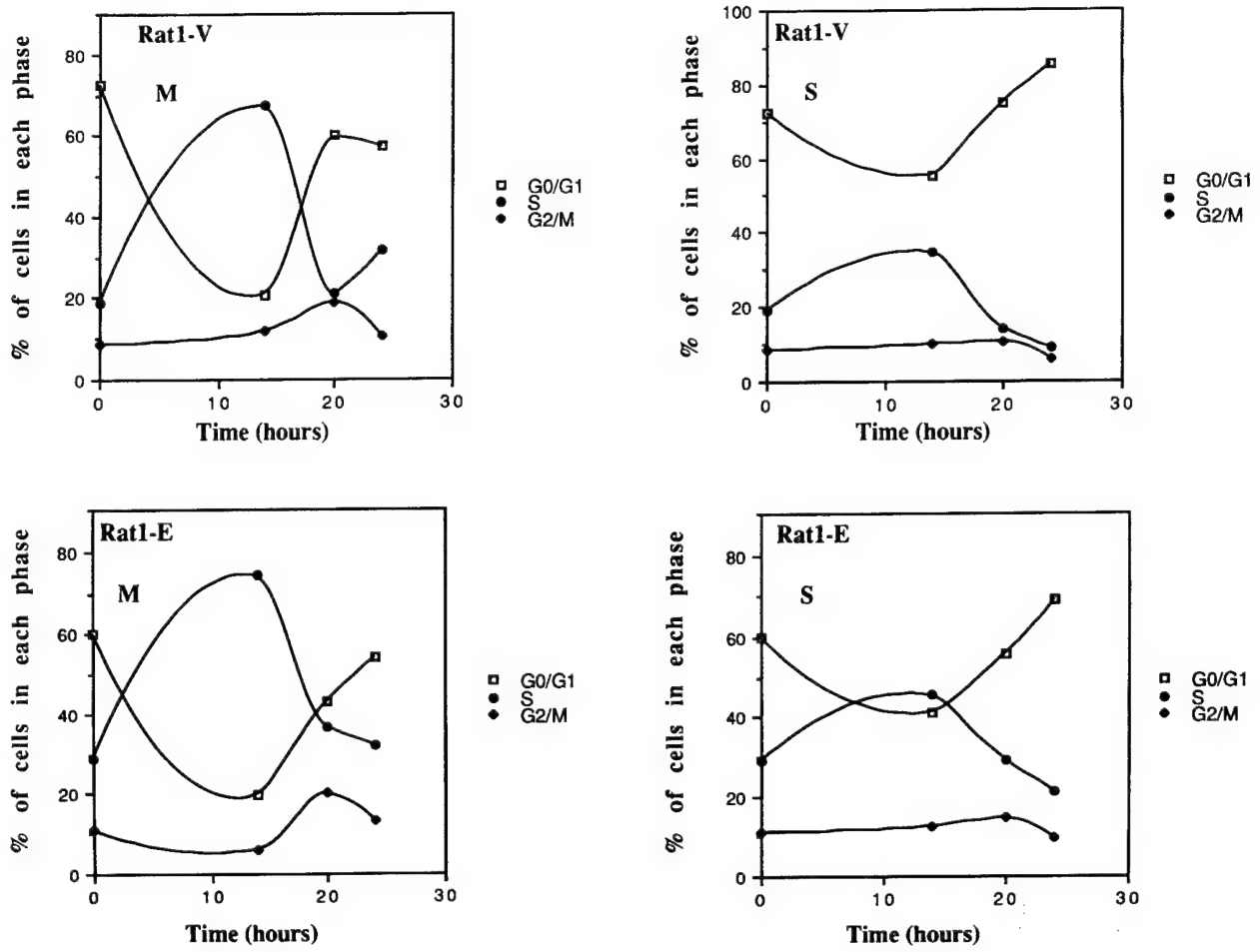

Fig. 4. Flow cytometric analysis of cell cycle progression in nonadherent cultures of vector control (Rat1-V) and cyclin E-overexpressing (Rat1-E) pools of Rat1 cells. Adherent cultures of cells were first synchronized in G_0 by serum starvation and then trypsinized and transferred in complete medium-containing serum into agar-coated dishes (*S*). Cells were subsequently collected at the indicated times, stained with propidium iodide, and analyzed by flow cytometry as described in "Materials and Methods." Parallel adherent cultures (*M*) of the same cell lines are shown for comparison. Cell viability in all cultures was >90% (by trypan blue exclusion) throughout the experiment. Similar results were obtained with several independent pools. *M,* monolayer; *S,* suspension.

at time 0 (Fig. 6*B, top panel, Lanes 1* and *6*). This finding was reproducible in several experiments. It might be because of the incomplete accumulation of the cells in G_0 (Fig. 4). However, the presence of cyclin E in fibroblasts arrested in G_0 by serum starvation has also been observed by other investigators (33, 34).

Assays for cyclin E-associated kinase activity indicated that this activity was maximal at about 14 h in the adherent cultures of both Rat1-PV and Rat1-PE cells, thus paralleling the level of expression of the cyclin E protein. Despite the high level of cyclin E protein, the cyclin E-associated kinase activity was much lower in the nonadherent cultures than the comparable adherent cultures. Therefore, in nonadherent cultures of Rat1-PV cells, there was only a slight increase in cyclin E-associated kinase activity at 14 h, and this activity could not be detected at 20 h. The induction of cyclin E-associated kinase activity was also reduced in the nonadherent cultures of Rat1-PE cells, when compared with monolayer cultures of the same cells. However, this activity was always higher in the nonadherent cultures of Rat1-PE cells than in nonadherent cultures of Rat1-PV cells, even at 20 h (Fig. 6*B*).

We then examined the level of the CDI $p27^{Kip1}$ under the same conditions. In the adherent cultures of both the Rat1-PV and Rat1-PE cells, the level of this protein at time 0 was relatively high (reflecting the effect of serum starvation). In both cell types, it was markedly decreased at 14 and 20 h (Fig. 6*B*). In the nonadherent cultures of both cell types, the level of $p27^{Kip1}$ remained high at both 14 and 20 h (Fig. 6*B*). This finding is consistent with previous studies on nonadherent KD and IMR90 cells (31).

These data demonstrate that although cyclin E overexpression is associated with an increased cyclin E-associated kinase activity in asynchronous adherent cultures of Rat1 cells, this increase is not sufficient to override the inhibition of cyclin E/Cdk2 activity that occurs in nonadherent cells. This apparently reflects the high levels of induction of $p27^{Kip1}$ in the nonadherent cultures.

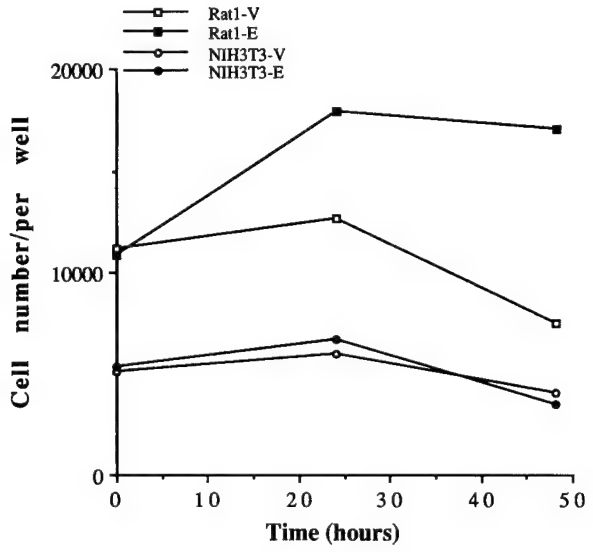

Fig. 5. Growth curves of nonadherent cultures of vector control (Rat1-V and NIH3T3-V) and cyclin E-overexpressing (Rat1-E and NIH3T3-E) pools of Rat1 and NIH3T3 cells. Asynchronous adherent cultures of each cell line were trypsinized, and the cells were replated into agar-coated dishes in complete growth medium containing serum. Cell numbers were determined in triplicate at the indicated times using a Coulter counter. No further growth was observed at later time points (data not shown). The data points are the means of triplicate assays that varied by less than 10% and represent the numbers of cells per 35-mm dishes. Cell viability in all cultures was >90%. Similar results were obtained with several independent pools.

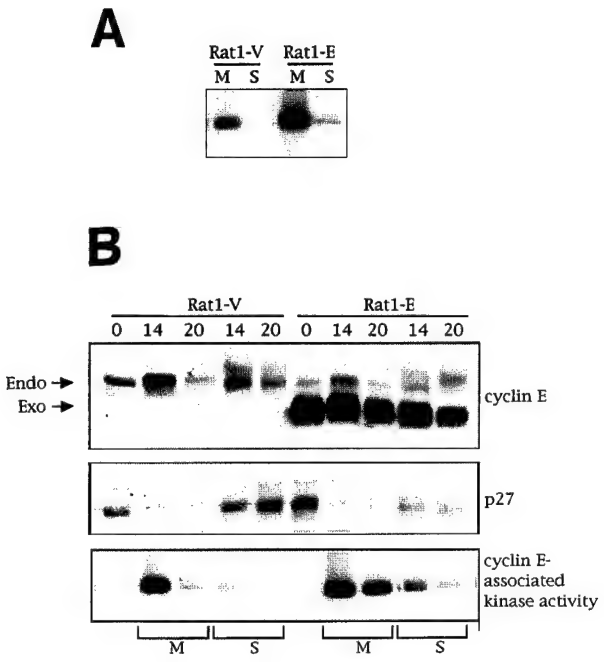

Fig. 6. Effects of cell adhesion on the level of expression of cyclin E and $p27^{Kip1}$ and on cyclin E-associated kinase activity in vector control (Rat1-V) and cyclin E-overexpressing (Rat1-E) pools of Rat1 cells. In *A*, 50 μg of whole-cell lysates from asynchronous adherent (*M*) and suspended (*S*) cultures of the indicated cell lines were used to analyze the histone H1 kinase activity of anti-cyclin E immunoprecipitates. In *B*, adherent cultures were first synchronized in G_0-G_1 by serum starvation. They were then trypsinized and replated as adherent (*M*) or suspension (*S*) cultures in the presence of serum. At the indicated times, cells were collected, and extracts were examined by Western blot analysis for cyclin E (*top panel*) and $p27^{Kip1}$ (*middle panel*) and for cyclin E-associated kinase activity using histone H1 as the substrate (*bottom panel*) as described in "Materials and Methods." The polyclonal anti-cyclin E antibody, which only recognizes the M_r 50,000 exogenous cyclin E band, was used for this experiment because it enabled us to detect both the exogenous and the endogenous cyclin E proteins. *Left,* positions of the endogenous (*Endo*) and the exogenous (*Exo*) cyclin E proteins. Similar results were obtained with several independent pools.

Overexpression of Cyclin E Modulates the Response of Some of These Cell Lines to TGF-β. TGF-β is a multifunctional cytokine that can affect cell proliferation and differentiation in a variety of cell types. It is a potent growth inhibitor of many types of normal epithelial cells, both in cell culture and *in vivo* (35). In some cell lines, the growth-inhibitory effects of TGF-β are associated with inhibition of cyclin E/Cdk2 kinase activity, although the mechanisms that mediate this inhibition appear to be different in different cell types (reviewed in Refs. 36 and 37). Therefore, it was of interest to determine whether overexpression of cyclin E modified the effects of TGF-β on cell proliferation.

HC11 cells are known to be responsive to the growth-inhibitory effects of TGF-β1 in terms of DNA synthesis and cell growth (38). Control HC11 cells and cyclin E-overexpressing derivatives were incubated in medium with or without TGF-β1 (5 ng/ml) for 48 h, and the cultures were then pulse labeled with [^{3}H]thymidine to measure DNA synthesis (see "Materials and Methods"). Fig. 7*A* indicates that TGF-β1 inhibited [^{3}H]thymidine incorporation by about 50% in both the parental and vector control cells. However, DNA synthesis was not inhibited in the HC11-E#8 cells that express a high level of cyclin E (Fig. 2; Ref. 28) but was inhibited by about 34% in the HC11-E#12 cells that express only a moderate level of cyclin E (28). Similar results were obtained after incubation with TGF-β1 for 24 or 72 h (data not shown). Growth curves in the presence of 5 ng/ml TGF-β1 with these four derivatives of HC11 cells confirmed that overexpression of cyclin E partially protected HC11 cells from inhibition by TGF-β1 (data not shown).

Overexpression of cyclin E in the 184B5 cell line partially protected them from inhibition of [^{3}H]thymidine incorporation by TGF-β (Fig. 7*B*) and also from inhibition of cell growth by TGF-β (data not shown), but this protection was not as great as that obtained with HC11 cells (Fig. 7*A*). This is probably because of the lower level of expression of the exogenous cyclin E in the 184B5 cells. Similar results were obtained after incubation with TGF-β1 for 24 or 72 h (data not shown). Under similar conditions, TGF-β1 caused about a 50% inhibition of [^{3}H]thymidine incorporation with both the parental and vector control of MCF-10F cells. However, with this cell line, a similar level of inhibition was observed with the cyclin E-overexpressing derivatives (Fig. 7*C*). Growth curves also indicated that TGF-β1 caused a similar extent of inhibition in both the vector control and cyclin E-overexpressing derivatives of MCF-10F cells (data not shown).

TGF-β1 stimulated [^{3}H]thymidine incorporation about 2.6-fold in both the Rat1 and NIH3T3 vector control fibroblasts. This is consistent with the known growth-stimulatory effects of TGF-β on fibroblasts (35, 39). This stimulation was re-

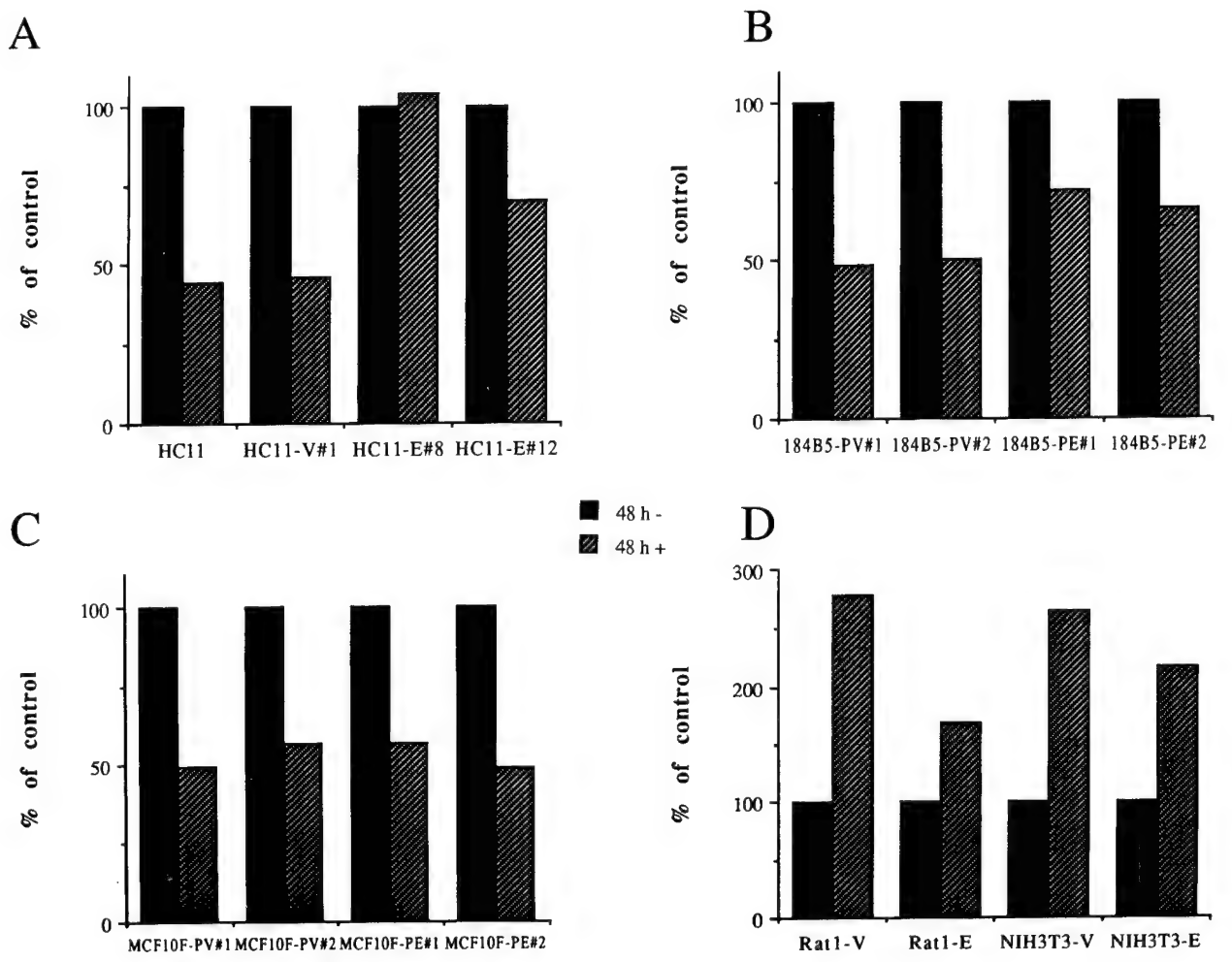

Fig. 7. Effects of stable overexpression of human cyclin E on the responses of different cell lines to TGF-β. Representative vector control (designated *V* or *PV*) and cyclin E-overexpressing derivatives (designated *E* or *PE*) of the indicated cell lines were plated in 24-well plates (10^4 cells/well), grown for 24–36 h, and then incubated in the absence or presence of TGF-β1 for 48 h (as described in "Materials and Methods") before adding [^{3}H]thymidine for 1 h. Values are expressed as the percentage of [^{3}H]thymidine incorporation into the acid-insoluble fraction in the presence of TGF-β1 (48 h+) *versus* the corresponding values obtained in the absence of TGF-β1 (48 h−), considered as 100%. Similar results were obtained after treating cells with TGF-β1 for 24 and 72 h (data not shown). Single clones were used for the HC11 cells, whereas pools of cells were used for all of the other cell lines. Individual points are the means of triplicate determinations. SDs for individual points were less than 20% of the mean. Similar results were obtained in three replicate experiments.

duced to 1.7- and 2.3-fold, respectively, in the corresponding cyclin E-overexpressing derivatives (Fig. 7*D*). Similar results were obtained after incubation with TGF-β1 for 24 or 72 h (data not shown). Therefore, overexpression of cyclin E can dampen the usual growth-inhibitory effects of TGF-β on epithelial cells and also dampen the usual growth-stimulatory effects of TGF-β on fibroblasts.

Discussion

Cyclin E overexpression has been observed in a variety of human cancers, including breast cancer, and it has been proposed that cyclin E might play an important role in tumor development, thus acting as an oncogene (25–27, 40). However, little is known about the actual role of increased expression of cyclin E in cell transformation and tumorigenesis. Therefore, it was of interest to examine, in parallel, the phenotypic effects of overexpressing an exogenous cyclin E in a series of cell lines.

The present study demonstrates that overexpression of the same cyclin E cDNA in different cell lines causes markedly different effects on cell cycle kinetics and growth parameters. These data are summarized in Table 1. Some of the data on HC11 cells were published previously (28) and are included for comparison. Overexpression of cyclin E was associated with a shortening of the G_1 phase of the cell cycle in Rat1 but not in NIH3T3 fibroblasts. Derivatives of both of these cell lines showed approximately similar increases in cyclin E-associated kinase activity, thus suggesting that high cyclin E/Cdk2 activity is necessary but not sufficient to drive entry into S phase. Both cell lines showed an increase in plating efficiency, but the saturation density was increased only in Rat1-PE cells when compared to the corresponding

vector control cells. Therefore, cyclin E overexpression caused an overall stimulation of growth in both of the fibroblast cell lines, but the characteristics and the magnitude of this stimulation were different. In mammary epithelial cells, increased expression of cyclin E was associated with a reduced percentage of cells in the G_0-G_1 phase of the cell cycle in the derivatives of the MCF-10F cells, whereas the cyclin E-overexpressing derivatives of 184B5 and HC11 cells displayed an increase in the percentage of cells in the G_0-G_1 phase of the cell cycle, and this was associated with a reduction of cells in the S phase. Cyclin E overexpression caused an overall stimulation of cell growth in MCF-10F cells but an inhibition of growth in 184B5 and HC11 cells. Unlike the situation in fibroblasts, these differences were directly related to the effects of cyclin E overexpression on cyclin E/Cdk2 activity. Therefore, cyclin E-associated kinase activity was increased in the MCF-10F but not in the HC11 and 184B5 derivatives. Derivatives of the latter two cell lines also displayed an increase in the level of expression of $p27^{Kip1}$, but the level of this protein was not increased in the cyclin E-overexpressing derivatives of MCF-10F, NIH3T3, and Rat-1 cells (Figs. 1 and 6). None of the cyclin E-overexpressing derivatives examined in this study displayed morphological evidence of malignant transformation or formed transformed foci in monolayer cultures.

It is of interest that the three cell lines, Rat1 fibroblasts, NIH3T3 fibroblasts, and MCF-10F cells, in which overexpression of cyclin E resulted in growth stimulation (by one or more parameters), also displayed increased cyclin E/Cdk2 kinase activity (Table 1). On the other hand, in the two cell lines 184B5 and HC11, in which cyclin E overexpression paradoxically caused an increase in the G_0-G_1 phase and an inhibition of cell growth, there was no increase in cyclin E/Cdk2 kinase activity, and there was increased cellular levels of $p27^{Kip1}$ protein. These differences among the cell types could not be explained simply by differences in the level of expression of the exogenous cyclin E cDNA (Fig. 2). Thus, although the 184B5 derivatives expressed a relatively low level of the exogenous cyclin E, their growth was inhibited, whereas the derivatives of MCF-10F and Rat1 cells expressed high levels of the exogenous cyclin E, and their growth was actually stimulated, although a comparable high level of exogenous cyclin E inhibited the HC11 cells (28). It would appear that in some, but not in all cell lines, increased expression of cyclin E can induce increased levels of the $p27^{Kip1}$ protein, which presumably accounts for the inhibitory effects seen in the latter cell lines. This effect is specific for $p27^{Kip1}$ because overexpression of cyclin E in the HC11 or MCF-10F cells and in Rat1 fibroblasts was not associated with altered levels of the CDIs $p21^{Waf1}$ and $p57^{Kip2}$ or cyclins D1 and A (28).[5] We have suggested previously that the increase in $p27^{Kip1}$ might reflect the existence of a homeostatic regulatory mechanism in which the set-point varies in different cell types (28). It may be of general relevance, because we have found that several human esophageal cancer cell lines,[5] breast cancer cell lines (28), and primary breast cancer cell lines,[5] express high levels of $p27^{Kip1}$ protein. The actual significance of this finding and the mechanism by which $p27^{Kip1}$ expression is induced is not known. It is of interest, however, that although the level of expression of $p27^{Kip1}$ in the three cyclin E-overexpressing pools of 184B5 cells was reproducibly higher than in control cells, it did not correlate quantitatively with the corresponding level of the exogenous cyclin E. In our previous studies on HC11 cells (28), we also found that although overexpression of cyclin E was associated with increased expression of $p27^{Kip1}$, there was not a proportional relationship between these two events. The significance of this finding is still unclear. It may be the effects of cyclin E overexpression on $p27^{Kip1}$ expression are not direct and because other still unknown factors influence the effects of cyclin E overexpression on $p27^{Kip1}$ expression. In studies on cyclin E-overexpressing derivatives of HC11 cells, we have found that the increased level of $p27^{Kip1}$ protein is not associated with an increase in $p27^{Kip1}$ mRNA, suggesting regulation at the protein level. Because recent studies indicate that the levels of $p27^{Kip1}$ protein can be regulated via a ubiquitin-dependent degradation pathway (41), it is possible that increased levels of cyclin E might interfere with this degradative mechanism in certain cell types. Indeed, in preliminary studies, we have found that the half-life of the $p27^{Kip1}$ protein is increased in the cyclin E-overexpressing HC11 cells. Studies are in progress to further examine the precise molecular mechanisms.

Recently, Fang *et al.* (31) reported the adhesion-dependence of cyclin E/Cdk2 activity in the KD and IMR90 untransformed human diploid fibroblast cell lines. They found that cyclin E/Cdk2 was activated in late G_1 in attached fibroblasts but not in fibroblasts maintained in suspension. In contrast, in the HUT12 cell line, an anchorage-independent, chemically transformed variant of KD cells, the cyclin E/Cdk2 complex was activated in late G_1 regardless of adhesion conditions. The lack of cyclin E/Cdk2 activity in suspended cells was associated with an increased expression of the Cdk2 inhibitors $p27^{Kip1}$ and $p21^{Waf1}$. Interestingly, these authors reported that the levels of expression of these Cdk2 inhibitors were comparable in both normal and transformed fibroblasts. Moreover, the levels of these two proteins increased in suspended cultures of transformed fibroblasts, as in the case of the normal fibroblasts. The maintenance of cyclin E/Cdk2 activity in suspended cultures of transformed fibroblasts was attributed to the higher levels of cyclin E, Cdk2, and the cyclin E/Cdk2 complex in these cells when compared to their normal counterparts. The authors concluded that the increased expression of cyclin E and Cdk2 may be important in the oncogenic transformation of HUT12 cells and suggested that the increased level of cyclin E might contribute to the anchorage-independent proliferation of tumor cells. Cyclin E overexpression has been reported in several human tumors, and its level has been related to tumor stage and grade in breast cancer (26). Moreover, it has been shown that the deregulated expression of cyclin E in some breast cancer cell lines is associated with an increased cyclin E-associated kinase activity, which unlike normal cells, is present constitutively and remains high throughout the cell cycle (27). Therefore, it was possible that cyclin E overex-

[5] Unpublished data.

pression contributed to tumor development by inducing anchorage-independent growth, which is a hallmark of transformed cells.

However, the present studies indicate that cyclin E overexpression is not sufficient *per se* to overcome the G_1 block observed in cells forced to grow in suspension. Thus, the growth of the cyclin E overexpressor NIH3T3-PE cells, like the vector control cells, was readily arrested in G_1 when they were deprived of adhesion. This G_1 block was delayed in the cyclin E-overexpressing derivatives of Rat1 cells, compared with vector control cells, because Rat1-PE cells showed a gradual and more delayed accumulation of cells in G_1 when kept in suspension culture. The limited G_1-S transit observed with the cyclin E-overexpressing derivatives of Rat1 cells was confirmed by doing growth curves on the cells in nonadherent cultures. Rat1-PE cells showed a significant increase in cell number after the first 24 h but no further increase at later time points, whereas no increase in cell number was observed in nonadherent cultures of Rat1-PV, NIH3T3-PV, and NIH3T3-PE cells (Fig. 5). These results suggest that overexpression of cyclin E in Rat1 fibroblasts, but not in NIH3T3 cells, confers only a very limited capacity for anchorage-independent growth, but it is not sufficient to completely override the adhesion requirement for continuous growth. Moreover, none of the cell lines used in this study were able to form colonies in soft agar after ectopic expression of cyclin E. Therefore, our data are not consistent with the suggestion of Fang *et al.* (31) that lack of cyclin E/Cdk2 kinase activity is the major factor responsible for the cell cycle block observed when nontransformed cells are grown in suspension. Our findings are, however, consistent with those of Guadagno *et al.* (42), indicating that lack of activation of cyclin A-associated kinase activity plays a critical role in this process. These investigators found that the expression of cyclin A in late G_1 was dependent on cell adhesion and that ectopic expression of cyclin A resulted in anchorage-independent growth in NRK fibroblasts. The fact that we found high levels of cyclin E in the arrested cells confirms that the block in cell cycle progression occurs at some point in late G_1 subsequent to cyclin E induction, probably before the induction of cyclin A and cyclin A/Cdk2 activity. It is, of course, possible that the lack of either cyclin E- or cyclin A-associated kinase activity is sufficient to cause the G_1 block observed in cells deprived of adhesion. Because our cyclin E overexpressor derivatives expressed levels of cyclin E that were often similar to those in human tumor cell lines (28) and the former cells were not anchorage independent, it seems likely that multiple events, in addition to cyclin E overexpression, contribute to the anchorage-independent growth observed with tumor cells. During the preparation of this manuscript, Zhu *et al.* (34) also reported that overexpression of cyclin E is not sufficient to induce anchorage-independent growth in human fibroblasts and also provided evidence that multiple events regulate anchorage-dependent growth.

It is well known that TGF-β is a complex regulator of cell growth (35). It displays activity on a great variety of cells and is able to either stimulate or inhibit their replication, depending on the cell type. The effects of TGF-β on the proliferation of several cell lines, both in terms of growth stimulation and growth inhibition, have been associated with the ability of this growth factor to interfere with the activity of the cyclin E/Cdk2 complex (reviewed in Refs. 36 and 37). Therefore, TGF-β causes a G_1 growth arrest in a variety of epithelial cell lines by inhibiting activation of the cyclin E/Cdk2 complex, although the mechanisms responsible for this inhibition appear to be different in different cell types and include: reduced expression of cyclin E; reduced expression of Cdk4; increased expression of p15; and increased expression of $p27^{Kip1}$ or $p21^{Waf1}$ (36, 37, 43). On the other hand, TGF-β stimulates the growth of fibroblast cell lines, and in some of these cell lines, this effect is associated with increased activity of the cyclin E/Cdk2 complex because of down-regulation of $p27^{Kip1}$ (44). It was of interest, therefore, to analyze whether overexpression of cyclin E was able to affect the responses of these cells to TGF-β in our model systems.

Indeed, in the present studies, we obtained preliminary evidence that overexpression of cyclin E interferes with the responsiveness of cells to TGF-β-mediated regulation of cell growth. Thus, in fibroblasts, in which TGF-β stimulates cell growth by activating cyclin E/Cdk2 activity (44), an increase in the basal level of cyclin E and cyclin E-associated kinase activity reduced the further stimulation of cell growth observed after exposure to TGF-β (Fig. 7*D*). The results obtained with the mammary epithelial cell lines were also of interest. Overexpression of cyclin E had no effect on TGF-β-mediated inhibition of growth in MCF-10F cells, but overexpression of cyclin E in HC11 and 184B5 cells reduced their responses to TGF-β-mediated inhibition of growth and DNA synthesis. This loss of inhibition was proportional to the level of expression of the exogenous cyclin E in both the HC11 and 184B5 cell lines and was not because of autonomous expression of TGF-β because immunoblot analysis with a pan-specific TGF-β antibody (R&D Systems, Minneapolis, MN) failed to detect TGF-β expression in either the control or the cyclin E overexpressor derivatives of these two cell lines (data not shown). Furthermore, the conditioned medium from the cyclin E-overexpressing cells had no effect on the rate of DNA synthesis or growth of the vector control cells, thus excluding that the cyclin E-overexpressing cells produce a soluble growth inhibitor (data not shown). Because there is evidence that TGF-β inhibits the growth of epithelial cell lines by reducing cyclin E/Cdk2 activity, the decreased inhibition of growth by TGF-β in the derivatives of HC11 and 184B5 cells may reflect the fact that they express increased levels of $p27^{Kip1}$ and already display decreased cyclin E/Cdk2 kinase activity. On the other hand, the decreased stimulation of growth by TGF-β in derivatives of fibroblasts that express increased levels of cyclin E may be because their high level of cyclin E kinase activity reduces the margin for a further TGF-β-mediated increase of this activity.

Regardless of the specific molecular mechanisms, the increased resistance to the TGF-β-mediated inhibition of cell growth seen with cyclin E-overexpressing derivatives of two mammary epithelial cell lines might be relevant to tumor development *in vivo*. This property could give a selective growth advantage to the cyclin E-overexpressing cells, although they grow more slowly than the parental cells. It is

tempting to speculate, therefore, that, at least in some cases, the frequent dysregulation of cyclin E in human tumor cells (26, 40) confers on these cells a selective advantage, not because it accelerates their cell cycle progression but because it increases their resistance to inhibition by TGF-β or other host factors. This is consistent with the observation that overexpression of cyclin E in primary tumors is not always associated with increased cell proliferation *in vivo* (26).

In conclusion, this report provides further evidence that the phenotypic effects of cyclin E overexpression are highly dependent on cell context and might depend on the interaction with other, not yet defined, pathways of cell regulation. Furthermore, we have also shown that overexpression of cyclin E in nontransformed fibroblasts or epithelial cells is not sufficient to confer anchorage-independent growth, although it might act in concert with other factors to cause this effect. Finally, we have demonstrated that overexpression of cyclin E can dampen the growth stimulatory effects of TGF-β in fibroblasts and the growth-inhibitory effects of TGF-β in mammary epithelial cells.

Although the underlying molecular mechanisms remain to be elucidated, the findings in this report indicate that it may not be possible to predict the specific phenotypic effects of increased expression of cyclin E in diverse human tumors, simply by measuring only the level of the cyclin E protein. Cell context also plays a major role presumably because of variations between cell types in diverse pathways that interact with the cyclin E/Cdk2 pathway.

Materials and Methods

Cells and Cell Culture. The origin and cell culture conditions of the normal human and mouse mammary epithelial cell lines 184B5 and HC11, respectively, and of the Rat1 fibroblasts have been described previously (28, 29). The MCF-10F, spontaneously immortalized human mammary epithelial cell line (45) was routinely cultured in a 1:1 (v/v) mixture of DMEM and Ham's F-12 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 0.5 μg/ml hydrocortisone, 0.1 μg/ml cholera toxin, 10 μg/ml insulin, 20 ng/ml epidermal growth factor (all from Sigma Chemical Co., St. Louis, MO), and 5% horse serum (Life Technologies, Inc.). NIH3T3 cells were cultured in DMEM medium plus 10% calf serum. The exponential doubling times and saturation densities of the various cell lines were determined essentially as described previously (28). Cells were plated at a density of 1×10^4 cells per 35-mm-diameter well, in triplicate. Every 2 days, the cultures were refed with fresh medium, and the number of cells per well was determined using a Coulter counter. The doubling times were calculated from the initial exponential phase of the growth curves and the saturation densities from the plateau of the growth curves. Growth in soft agar was also performed as described previously (28). For the bottom layer of agar, 1 ml of 0.5% agar was placed in each 35-mm well of six-well plates. Then, 2 ml of 0.3% agar containing 1×10^5 cells were layered on top of the solidified layer of bottom agar. Colony formation was monitored by microscopy for up to 14 days, and the final numbers of colonies larger than 0.1 mm in diameter were determined. Plating efficiency assays were performed by seeding 1000 cells per 10-cm dish in complete medium. Cells were refed with fresh medium every 3–4 days for about 2 weeks. The cells were then fixed and stained with Giemsa, and the number of grossly visible colonies was counted.

All assays were performed in triplicate, and all experiments were repeated at least twice for all of the above-reported derivatives. The data reported in Table 1 are the results of a typical experiment for a representative cell line.

Construction of Retrovirus Vectors and Viral Transduction. The construction of the cyclin E retroviral expression plasmid PMV12-cycE and the method used for retrovirus packaging and transduction have been described previously (28). Briefly, to prepare infectious retrovirus particles, the PMV12-cycE plasmid or the control vector PMV12pl were transfected into the Ψ2 ecotropic (46) or the ΨAM amphotropic retrovirus packaging cell lines. The transfected cells were selected by growth in hygromycin (Boehringer Mannheim, Indianapolis, IN), and the cell-free media containing defective recombinant viruses were harvested, filtered, and used for the infection. Following selection for cells resistant to hygromycin (*hph*), several pools of thousands of resistant colonies were obtained and used for further analysis.

Characterization of Anchorage-independent Growth. G_0-synchronized cultures of NIH3T3 and Rat1 fibroblasts were prepared by incubation of subconfluent cultures in serum-free medium (DMEM supplemented with 5 μg/ml transferrin, 10 μg/ml bovine insulin, and 5 mg/ml BSA) for 48 or 72 h, respectively. Control studies showed that cells were >95% viable after this period, as assessed by trypan blue exclusion. Parallel adherent and nonadherent cultures were prepared according to a protocol described previously (32). Briefly, G_0-synchronized monolayers were trypsinized and replated into untreated (adherent cultures) or agar-coated (nonadherent cultures) tissue culture plastic dishes (6 ml of serum-free medium containing 1% agar in 10-cm dishes) in serum-free medium. After 6–10 h incubation (to allow cells in adherent cultures to adhere to the plate), calf serum was added to both adherent and nonadherent cultures (final concentration, 10%). Cells were collected at the indicated times by centrifugation and processed for flow cytometry or immunoblotting analyses. Alternatively, asynchronous, adherent cultures were trypsinized and replated into untreated or agar-coated dishes in medium containing 10% serum to obtain parallel adherent (monolayer) and nonadherent (suspension) cultures. For growth curves, cells were plated into untreated or agar-coated 35-mm dishes in complete medium, and 2 ml of fresh medium were added to the cultures every 2 days.

Flow Cytometric Analysis. Cells from parallel adherent and nonadherent cultures were collected by centrifugation, either directly (suspended cultures) or after trypsinization (adherent cultures) and washed twice with PBS. Cell pellets were resuspended in 1 ml PBS and fixed in 5 ml of 70% ethanol and stored at 4°C. For the analysis, cells were collected by centrifugation, and the pellets were resuspended in 0.2 mg/ml of propidium iodide in HBSS containing 0.6% NP40. RNase (1 mg/ml; Boehringer Mannheim) was added, and the suspension was incubated in the dark at room temperature for 30 min. The cell suspension was then filtered through a 41 μm Spectra mesh filter (Spectrum, Houston, TX) and analyzed for DNA content on a Coulter EPICS 753 flow cytometer. The percentage of cells in different phases of the cell cycle was determined using a ModFit 5.2 computer program.

DNA Synthesis. Cells were plated in triplicate in 24-well plates (10^4 cells/well) in the appropriate medium and grown for 24–36 h. The media were then changed, and the cells were incubated in the absence or presence of TGF-β1 (R&D Systems, Inc., Minneapolis, MN). At the indicated time points, the cultures were labeled for 1 h with [^{3}H]thymidine (1 μCi/ml; (Amersham Corp., Arlington Heights, IL) and then washed with ice-cold PBS and extracted with 10% cold trichloroacetic acid for 15 min on ice. After solubilization in 0.5 N NaOH, trichloroacetic acid-insoluble radioactivity was determined by liquid scintillation counting. Five ng/ml TGF-β1 in regular medium were used for the epithelial cell lines, whereas 10 ng/ml and 2 ng/ml in DMEM with 2% calf serum were used for Rat1 and NIH3T3 fibroblasts, respectively. Values are expressed as the percentage of [^{3}H]thymidine incorporation into the acid-insoluble fraction in the presence of TGF-β1 *versus* control values in the absence of TGF-β1. Qualitatively similar results were obtained after 24 and 72 h (data not shown).

Immunoreagents. The polyclonal antibody to cyclin E was obtained from Upstate Biotechnology (Lake Placid, NY). The monoclonal antibodies to cyclin E were purchased from PharMingen (San Diego, CA). The polyclonal antibody to $p27^{Kip1}$ was from Santa Cruz Biotechnology (Santa Cruz, CA).

Immune Complex Kinase Assay and Immunoblotting. Cdk enzyme assays were performed as described previously (28). Cell pellets were resuspended in kinase-lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1% Tween 20, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM sodium orthovanadate, 10 mg/ml leupeptin, and 10 mg/ml aprotinin (all of these chemicals were from Sigma)] and sonicated twice using a Sonifier cell disruptor (Ultrasonics, Inc., Plainview, NY). After centrifugation, clarified materials (50 mg) were incubated with protein A-Sepha-

rose for 1 h at 4°C for preclearing in IP buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, and 0.1% Tween 20]. Immunoprecipitations were carried out with 2 μg of the indicated antibody, and immunocomplexes were recovered with protein A-Sepharose. For H1 kinase activity, the protein A beads were washed four times with IP buffer, twice with washing buffer [50 mM HEPES (pH 7.5) and 1 mM DTT], and once with kinase buffer [50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM NaF, and 0.1 mM sodium orthovanadate]. The final pellet was resuspended in 50 ml of kinase buffer supplemented with 2 μg of histone H1 (Boehringer Mannheim) and 5 μCi of [γ-^{32}P]ATP (Amersham) and incubated for 15 min at 30°C. The reaction was stopped by the addition of 25 μl of 2×-concentrated Laemmli sample buffer. The samples were separated on a SDS-10% polyacrylamide gel, and the phosphorylated histone H1 was visualized by autoradiography. Proteins from total cell lysates (50 μg) were used for Western blot analysis as described previously (28). Samples were electrophoresed by SDS-PAGE and then transferred to Immobilon-P membranes (Millipore, Bedford, MA). Blots were then incubated with blocking buffer (50 mM Tris, 200 mM NaCl, 0.2% Triton X-100, and 3% BSA) for 90 min at room temperature. Immunodetection was performed using the enhanced chemiluminescence (ECL) kit for Western blotting detection (Amersham).

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Deregulated Expression of $p27^{Kip1}$ in Human Breast Cancers[1]

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ABSTRACT

Protein complexes composed of cyclins and cyclin-dependent kinases control the orderly progression of mammalian cells through the cell cycle. The $p27^{Kip1}$ protein belongs to a family of cyclin-dependent kinase-inhibitory proteins that are negative regulators of cell cycle progression and have been proposed as candidate tumor suppressor genes. However, the *$p27^{Kip1}$* gene is only rarely mutated in human primary breast carcinomas and breast cancer cell lines. To further address the role of *$p27^{Kip1}$* in the development of human tumors, we determined by Western blot analysis the levels of expression of the $p27^{Kip1}$ protein in a series of human cancer cell lines and found that this protein is expressed at high levels in many of these cell lines, even during exponential growth. The levels of $p27^{Kip1}$ were significantly associated with the levels of cyclins D1 and E. In contrast to the high level of $p27^{Kip1}$ in breast cancer cell lines, three cell lines established from normal mammary epithelium expressed low levels of this protein. Cell synchronization studies demonstrated deregulation of the expression of $p27^{Kip1}$ throughout the cell cycle in two breast cancer cell lines but normal regulation in a normal mammary epithelial cell line. Immunohistochemical studies on $p27^{Kip1}$ expression in 52 primary human breast cancers indicated that this protein was also expressed at relatively high levels in 44% of the tumor samples, but it was barely detectable or undetectable in the remaining 56% of the samples. Additional studies are required to determine why some breast cancer cells express relatively high levels of $p27^{Kip1}$ despite its known role as an inhibitor of cell cycle progression.

INTRODUCTION

Cyclins control the orderly progression of cells through the cell cycle by determining the timing of activation and the substrate specificity of a series of Cdks,[3] which are sequentially activated during specific phases of the cell cycle. G_1 cyclins regulate the progression of cells through the G_1 phase and drive entry into the S phase. Three D-type cyclins (cyclin D1–3) act at mid-G_1 by complexing with either Cdk4 or Cdk6. Cyclin E acts in late G_1 by complexing with Cdk2 (reviewed in Refs. 1–3).

Both positive and negative phosphorylation events, as well as specific inhibitory proteins, play critical roles in regulating the activation of cyclin/Cdk complexes during cell cycle progression (4, 5). The CDIs identified in mammalian cells are classified into two major categories. The INK4 family includes $p16^{Ink4a}$ (6), $p15^{Ink4b}$ (7), $p18^{Ink4c}$ (8), and $p19^{Ink4d}$ (9), which mainly inhibit Cdk4 and Cdk6 by binding to the Cdk subunit itself. The Cip/Kip family includes $p21^{Cip1}$ (10), $p27^{Kip1}$ (11), and $p57^{Kip2}$ (12), which share a conserved domain (13) and inhibit a broader range of Cdks by binding to several cyclin/Cdk complexes (3–5). All of these CDIs cause G_1 arrest when overexpressed in transfected cells (see Refs. 3–5 for review).

Because the CDIs are potent negative regulators of the cell cycle, it has been suggested that they may also function as tumor suppressor genes and that their loss plays an important role in the development of human tumors. Indeed, loss of $p15^{Ink4b}$ and $p16^{Ink4a}$ expression due to mutation, deletion, and/or methylation of the corresponding genes has been seen in a variety of human tumors (14–16), and $p16^{Ink4a}$-deficient mice develop spontaneous tumors at an early age and are highly sensitive to carcinogens (17). On the other hand, several independent studies have found that alterations in the integrity of human *$p27^{Kip1}$* (18–24) and *$p21^{Cip1}$* (25) genes occur only rarely in a variety of human primary tumors and cancer cell lines (24). It has been hypothesized that mutations and/or inactivation of these genes might be catastrophic, resulting in cell death. Thus, low levels of $p21^{Cip1}$ and $p27^{Kip1}$ might be necessary for normal cell cycle progression, whereas higher levels might be required for growth-inhibitory pathways. However, this hypothesis has not been confirmed by the results obtained with knockout mice. In fact, $p21^{Cip1}$-deficient mice undergo normal development, although fibroblasts from these mice are defective in G_1 arrest in response to DNA damage and nucleotide pool depletion (26, 27). Moreover, unlike the p53-deficient mice, $p21^{Cip1}$-deficient mice do not exhibit a marked increase in spontaneous tumors (27). The $p27^{Kip1}$-deficient mice complete an apparently normal

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[3] The abbreviations used are: Cdk, cyclin-dependent kinase; CDI, Cdk inhibitor; Rb, retinoblastoma.

prenatal development, although they develop hyperplasia in multiple organs, retinal dysplasia, and pituitary tumors (28–30). Nevertheless, fibroblasts from these mice display a normal G_1 arrest in response to a variety of extracellular stimuli (30).

Although $p27^{Kip1}$ is associated predominantly with cyclin D/Cdk4 (11, 31) or cyclin D/Cdk6 (32) and is found in Cdk/cyclin complexes throughout the G_1 phase of the cell cycle, it appears that the cyclin E/Cdk2 complex is the major target of its inhibitory activity (33, 34). The expression of cyclin E is altered both qualitatively and quantitatively in breast cancer cell lines. Thus, when compared with normal human mammary epithelial cells, breast cancer cell lines often express increased levels of multiple cyclin E protein bands that range in size from M_r 35,000–50,000 (35, 36). Moreover, the level of cyclin E is not cell cycle regulated and remains in a catalytically active complex throughout the cell cycle in the breast cancer cell line MDA-MB-157 (37).

We reported recently that overexpression of cyclin E in a mouse mammary epithelial cell line is associated with increased expression of $p27^{Kip1}$ (38). Similar results were obtained with a normal human mammary epithelial cell line.[4] It has been recently reported that increased expression of $p27^{Kip1}$ might also induce increased expression of cyclin E by inhibiting its phosphorylation by Cdk2, which is essential for the ubiquitin-mediated degradation of cyclin E (39). Other observations have also suggested a possible relationship between the levels of expression of cyclin D1, Rb and $p27^{Kip1}$ (40, 41).[5]

In the present study, we found that $p27^{Kip1}$ is expressed at a relatively high level in a variety of human cancer cell lines and in a major fraction of primary human breast cancers. We also found that the cell cycle regulation of $p27^{Kip1}$ is altered in breast cancer cells compared to normal mammary epithelial cells. The possible implications of these findings are discussed.

MATERIALS AND METHODS

Cells and Cell Culture. The spontaneously immortalized human mammary epithelial cell line MCF-10F (42) was cultured routinely in a 1:1 (vol:vol) mixture of DMEM and Ham's F12 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 0.5 μg/ml hydrocortisone, 0.1 μg/ml cholera toxin, 10 μg/ml insulin, 20 ng/ml epidermal growth factor (all from Sigma Chemical Co., St. Louis, MO), and 5% horse serum (Life Technologies). The immortalized human mammary epithelial cell lines 184B5 and 184A1 were grown in supplemented MCDB 170 medium (Clonetics Corp., San Diego, CA), as described previously (38, 43). All of the other cell lines used in this study were obtained from the American Type Culture Collection and were cultured as recommended by the supplier. A previously published protocol was used to synchronize the cancer cell lines using phenol red-free medium (44). DNA synthesis was monitored as described previously (38).

Immunoreagents. The polyclonal antibody to cyclin E was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY), the monoclonal antibodies to cyclin E and to Rb were obtained from PharMingen (San Diego, CA) and the polyclonal antibody to $p27^{Kip1}$ from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunoblotting and Cyclin E-associated Kinase Assay. Western blot analyses of total cell extracts and *in vitro* assays for cyclin E-associated histone H1 kinase activity were performed as described previously (38). The immunoprecipitates for the kinase assays were obtained with 2 μg of the polyclonal anti-cyclin E antibody, and immunocomplexes were recovered with protein A-Sepharose. Immunodetection was performed using the enhanced chemiluminescence kit for Western blots (Amersham Corp., Arlington Heights, IL). The intensities of each of the bands on the Western blots, as well as on the SDS-PAGE gels used to assess histone H1 kinase activity, were quantitated on a Molecular Dynamics (Sunnyvale, CA) computing densitometer, using Image Quant software (version 3.22). Correlations between two parameters were evaluated by linear regression. All assays on each cell line were repeated at least twice and gave similar results. The assays for detecting heat-stable CDK-inhibitory activity in protein extracts were performed as described previously (38).

Tissue Samples. Tumor samples were from patients who underwent routine surgery for breast cancer at the Columbia-Presbyterian Medical Center (New York, NY). Tumors were classified according to the American Joint Committee on Cancer system (45). Four cases were infiltrating lobular carcinomas, and the remaining were infiltrating or *in situ* ductal carcinomas. Other clinicopathological parameters such as age, tumor size, grading, regional lymph node involvement, vascular invasion, DNA index, S-phase fraction, estrogen and progesterone receptor status, and c-*erb*B-2 expression, were also recorded. The samples were coded, and the names of the patients were not revealed.

Immunohistochemistry. All immunohistochemical analyses were performed on routinely processed, formalin-fixed, paraffin-embedded tissues using an avidin-biotin complex immunoperoxidase technique. Five-μm tissue sections were mounted on poly-L-lysine-coated slides. After incubation in an oven at 58°C overnight to prevent sections from lifting from the slide during the antigen-retrieval step, sections were dewaxed and rehydrated. They were then submerged in 1 mM EDTA buffer (pH 8.0) and microwaved for a total of 10 min at 750 W. We first determined that these conditions were optimal for antigen retrieval and found that they gave better results than using a citrate buffer (10 mM, pH 6.0). Sections were then treated to block endogenous peroxidase, and after blocking with goat serum for 1 h at room temperature, the primary antibody, a polyclonal IgG-rabbit antihuman $p27^{Kip1}$ antibody (C-19; Santa Cruz), was applied overnight at 4°C in a high-humidity chamber. This antibody was raised against a peptide corresponding to amino acids 181–198 in the COOH terminus of the human $p27^{Kip1}$ protein. The primary antibody was used at a concentration of 0.2 μg/ml (in PBS with 10% goat serum), because this gave good nuclear staining with minimal background. As a negative control, a duplicate section of each tissue sample was immunostained in the absence of the primary antibody. A breast carcinoma with known positive immunostaining for $p27^{Kip1}$ served

[4] A. Sgambato and I. B. Weinstein, unpublished data.

[5] Doki, Y., Imoto, M., Han, E. K-H., Sgambato, A., and Weinstein, I. B. Increased expression of the $p27^{KIP1}$ protein in human esophageal cancer cell lines that overexpress cyclin D1. Carcinogenesis, *18:* 1139–1148, 1997.

Table 1 $p27^{Kip1}$ immunostaining in primary human breast cancers[a]

	No.	%
Positive	23 of 52	44%
Negative	29 of 52	56%

[a] Positive designates tumors with 2 or 3+ immunostaining, and negative designates tumors with 0 or 1+ immunostaining. Thirteen of the 23 positive tumors were 3+. No significant association was observed between $p27^{Kip1}$ immunostaining and tumor stage ($P = 0.2$), tumor grade ($P = 0.6$), S-phase fraction (<15%, >15%; $P = 0.1$), DNA index ($P = 0.8$), estrogen ($P = 1.0$) and progesterone ($P = 1.0$) receptor status, c-*erb*B2 expression ($P = 1.0$), lymph node involvement ($P = 0.3$), and vascular invasion ($P = 0.1$), but we emphasize that this set of samples is relatively small.

as a positive control. After washing, immunostaining was performed using rabbit IgG Vectastain ABC and diaminobenzidine kits (Vector Laboratories), as described (46). A slightly modified protocol was used for cells cultured on glass slides, as described previously (38).

The specificity of the reaction was demonstrated by inhibition of the immunohistochemical staining in positive controls by preincubating the antibody with a 100-fold excess of the immunizing peptide for 1 h at room temperature (data not shown). Confirmatory results were obtained when a different antibody (N-20, Santa Cruz) was used to stain selected cases for which duplicate slides were available (data not shown). All slides were interpreted independently by two of the authors (A. S. and Y-J. Z.), using coded samples. The few cases with discrepant scoring were re-evaluated jointly on a second occasion, and agreement was reached. Staining intensity was graded as follows: no staining (0), weakly positive (1+), moderately positive (2+) and strongly positive (3+). Nonneoplastic epithelial cells, stromal cells, and inflammatory cells were also evaluated for $p27^{Kip1}$ immunostaining. Positive and negative control slides were included within each batch of slides.

The two-tailed Fisher's exact test was used to test for the association between $p27^{Kip1}$ expression and other clinicopathological variables (Table 1).

RESULTS

Elevated Expression of $p27^{Kip1}$ Correlates with Increased Expression of Cyclin E and Cyclin D1 in Human Cancer Cell Lines. To further address the role of $p27^{Kip1}$ in human cancers and its relationship with other regulators of the cell cycle machinery, we examined by Western blot analysis the levels of expression of this protein in a series of human cancer cell lines derived from various types of cancers. For comparison, we included three nontumorigenic human mammary epithelial cell lines (Fig. 1, *184A1, 184B5,* and *MCF-10F*) and a primary culture of normal human skin fibroblasts (passage 8; Fig. 1, *HSF*). All of these four normal (nontumorigenic) cell lines expressed negligible or very low levels of $p27^{Kip1}$. On the other hand, four breast cancer cell lines (Fig. 1, *MCF-7, T-47D, DU4475,* and *BT-549*) and the human mammary epithelial cell line HBL-100 (Fig. 1), which was established originally from apparently normal human mammary epithelium but expresses SV40 large T antigen (47) and is transformed partially (48), expressed high levels of this protein. The latter results were reported previously by us (38) and are shown here merely for comparison with the other cell lines.

Four of the six colon cancer cell lines examined (Fig. 1, *HT29, SW480-R2, HCT116,* and *LS1747*) also showed a relatively high level of expression of $p27^{Kip1}$, whereas only a slight increase was observed in the other two colon cancer cell lines (Fig. 1, *SW480-E8* and *SW620*). The level of $p27^{Kip1}$ was also high in one (Fig. 1, *LNCap*) of the three prostate cancer cell lines analyzed (Fig. 1, *PC-3, DU145,* and *LNCap*) but was low in two head and neck cancer cell lines (Fig. 1, *HSC3* and *HSC4*), an osteosarcoma (Saos-2) and a promyelocytic leukemia cell line (HL-60). In a separate study, we also observed high expression of $p27^{Kip1}$ in five of eight human esophageal cancer cell lines.[5]

We then examined by Western blot analysis the levels of expression of cyclin E, cyclin D1, and Rb in the same series of human cell lines. Most of the cell lines that expressed high levels of $p27^{Kip1}$ also expressed high levels of the cyclin E and/or cyclin D1 and Rb proteins. When the intensities of the corresponding bands were quantitated by densitometry and correlations between two parameters were evaluated by linear regression, the levels of $p27^{Kip1}$ showed a significant association with the levels of cyclin D1 ($P < 0.001$) and cyclin E ($P < 0.0001$) in this series of cell lines (Figs. 1 and 2). No significant correlation was observed with the levels of the Rb protein (Fig. 2) or with the CDIs $p16^{Ink4a}$, $p21^{Cip1}$, and $p57^{Kip2}$ (data not shown).

Impairment of Cell Cycle Regulation of $p27^{Kip1}$ in Breast Cancer Cells. To further investigate the significance of the increased expression of $p27^{Kip1}$ in tumor cells, we analyzed the cell cycle expression of this protein in the normal immortalized human mammary epithelial cell line MCF-10F, and in the breast cancer cell lines MCF-7 and T-47D that express a high basal level of $p27^{Kip1}$ (Fig. 1). We also analyzed the level of expression of cyclin E and of cyclin E-associated kinase activity in the same cell extracts. The three cell lines were synchronized by arresting them in G_0 by serum starvation, using previously published protocols (Ref. 44; see "Materials and Methods" for details). Cell synchronization was verified by fluorescence-activated cell sorting analysis, which confirmed that with all three cell lines, more than 85% of the cells were arrested in G_0/G_1 (data not shown). Analysis of DNA synthesis by [^{3}H]thymidine incorporation indicated that all three cell lines entered S phase at about 20 h after they were restimulated with serum (Fig. 3*C*). Cells were harvested at various times after restimulation with serum, and the extracted proteins were analyzed by Western blot analyses and by kinase assays. The pattern of expression of cyclin E in the MCF-10F cells was consistent with that reported previously for normal mammary epithelial cells (37), with levels rising slowly during the G_0-to-S transition (Fig. 3*A*). Cyclin E-associated kinase activity was also cell cycle regulated in these cells and coincided with the time of expression of the cyclin E protein, progressively rising after serum restimulation (Fig. 3*A*).

In contrast to the above results, when arrested at G_0, the two breast cancer cell lines MCF-7 and T-47D expressed high levels of multiple cyclin E-related protein bands, and the levels of these proteins remained high throughout the time course following restimulation with serum (Fig. 3*A*). Similar results

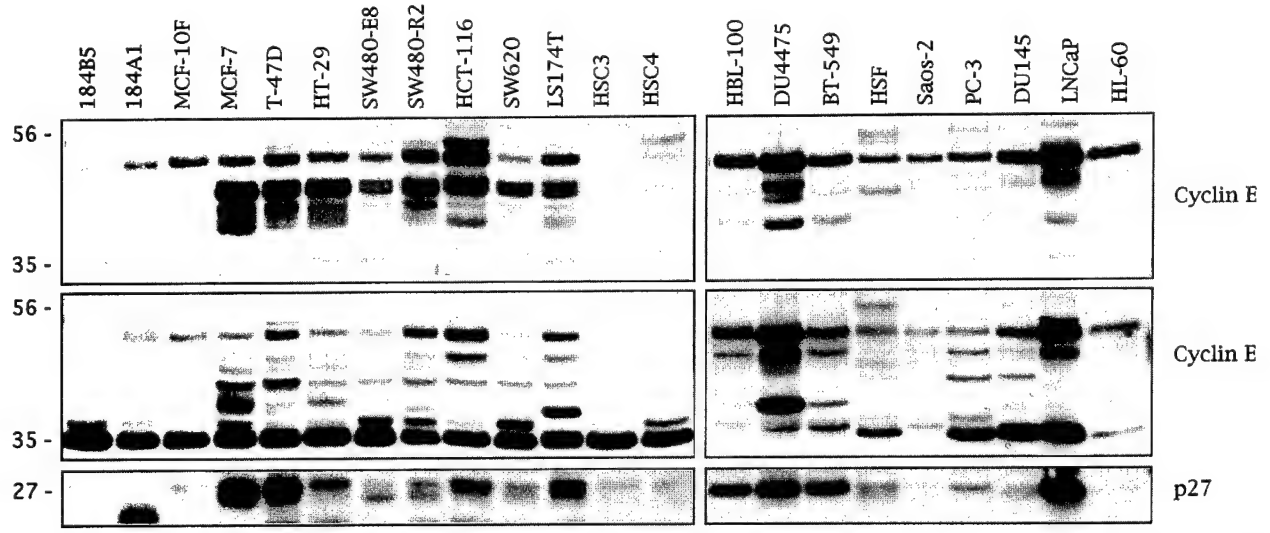

Fig. 1 Abnormal expression of $p27^{Kip1}$ and cyclin E in human cancer cell lines. Western blot analysis for cyclin E (*top* and *middle*) and $p27^{Kip1}$ (*bottom*) expression in: three normal immortalized nontumorigenic human mammary epithelial cell lines [*184A1, 184B5,* and *MCF-10F* (*left*)], four breast cancer cell lines [*MCF-7* and *T-47D* (*left*) and *DU4475* and *BT-549* (*right*)], and a SV40 large T-immortalized human mammary epithelial cell line, HBL-100 (*right*). Also shown are: six colon cancer cell lines [*HT-29, SW480-E8, SW480-R2, HCT-116, SW620,* and *LS174T* (*left*)], two head and neck cancer cell lines [*HSC3* and *HSC4* (*left*)], primary human skin fibroblasts [*HSF* (*right*)], an osteosarcoma cell line [*Saos-2* (*right*)], three prostate cancer cell lines [*PC-3, DU145,* and *LNCaP* (*right*)], and a promyelocytic leukemia cell line [*HL-60* (*right*)]. Whole-cell extracts were prepared from exponentially proliferating cultures of the indicated cell lines. Fifty μg of protein were resolved by SDS-PAGE and transferred to an Immobilon membrane. Duplicate blots were probed with a monoclonal antihuman cyclin E antibody (*top*), a polyclonal anticyclin E antibody (*middle*), and a polyclonal anti-$p27^{Kip1}$ antibody (*bottom*), respectively. Faint bands corresponding to the M_r 50,000 and 42,000 forms of cyclin E proteins were visible in the normal cell lines after longer exposure to the X-ray film. For additional details, see "Materials and Methods."

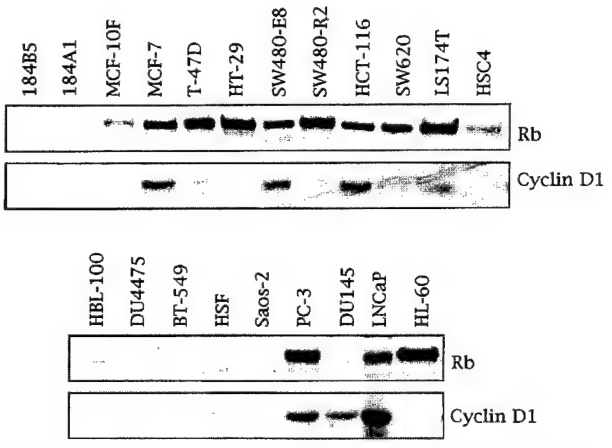

Fig. 2 Abnormal expression of cyclin D1 and Rb proteins in human cancer cell lines. Duplicate blots were probed with a monoclonal antihuman Rb or a polyclonal antihuman cyclin D1 antibody, respectively.

have been reported for the MDA-MB-157 breast cancer cell line, in which the *cyclin E* gene is actually amplified (35, 37), but the overexpression of cyclin E in MCF-7 and T-47D cells is not due to gene amplification (35). Nevertheless, cyclin E-associated kinase activity showed a progressive increase from time 0 to 20 h following restimulation of the MCF-7 cells with serum, thus resembling the finding with the MCF-10F cells. The T-47D cells displayed a relatively high level of cyclin E-associated kinase activity at time 0 and only about a 2-fold increase at 20 h (Fig. 3, *A* and *B*).

The expression of $p27^{Kip1}$ was cell cycle regulated in the normal mammary epithelial cell line MCF-10F (Fig. 3*A*). Thus, it was expressed at a high level in serum-starved G_0 cells (time 0), and its level decreased rapidly after serum restimulation (within 4 h; data not shown). It was almost undetectable after about 12 h and remained at this low level throughout the remaining time course, up to 32 h (Fig. 3*A*). Densitometric analysis indicated that the decrease in the level of $p27^{Kip1}$ was about 100-fold during this time course (Fig. 3*B*). However, with the two breast cancer cell lines MCF-7 and T-47D, the level of expression of $p27^{Kip1}$, which was very high in the G_0-arrested MCF-7 cell line and moderately high in the T-47D cell line, remained at a constant level throughout the time course of serum restimulation or showed only a slight reduction (less than 1.5–2-fold; Fig. 3, *A* and *B*).

In addition to the major M_r 27,000 band, the anti-$p27^{Kip1}$ antibody sometimes recognized an additional fainter, slowly migrating band (Fig. 3*A*), the presence of which depended on the lysis buffer used to prepare the cell extracts (data not shown). This band probably corresponds to a phosphorylated form of the $p27^{Kip1}$ protein, because it disappeared after treatment of the cell extracts with phosphatase (data not shown). A second $p27^{Kip1}$ band was also detected by other investigators using the same antibody (49).

To investigate whether the differences in $p27^{Kip1}$ expression between the normal and cancer cell lines were associated with changes in the subcellular localization of $p27^{Kip1}$, we carried out immunostaining with the anti-$p27^{Kip1}$ antibody on nonsynchronized cultures of the MCF-10F and MCF-7 cell

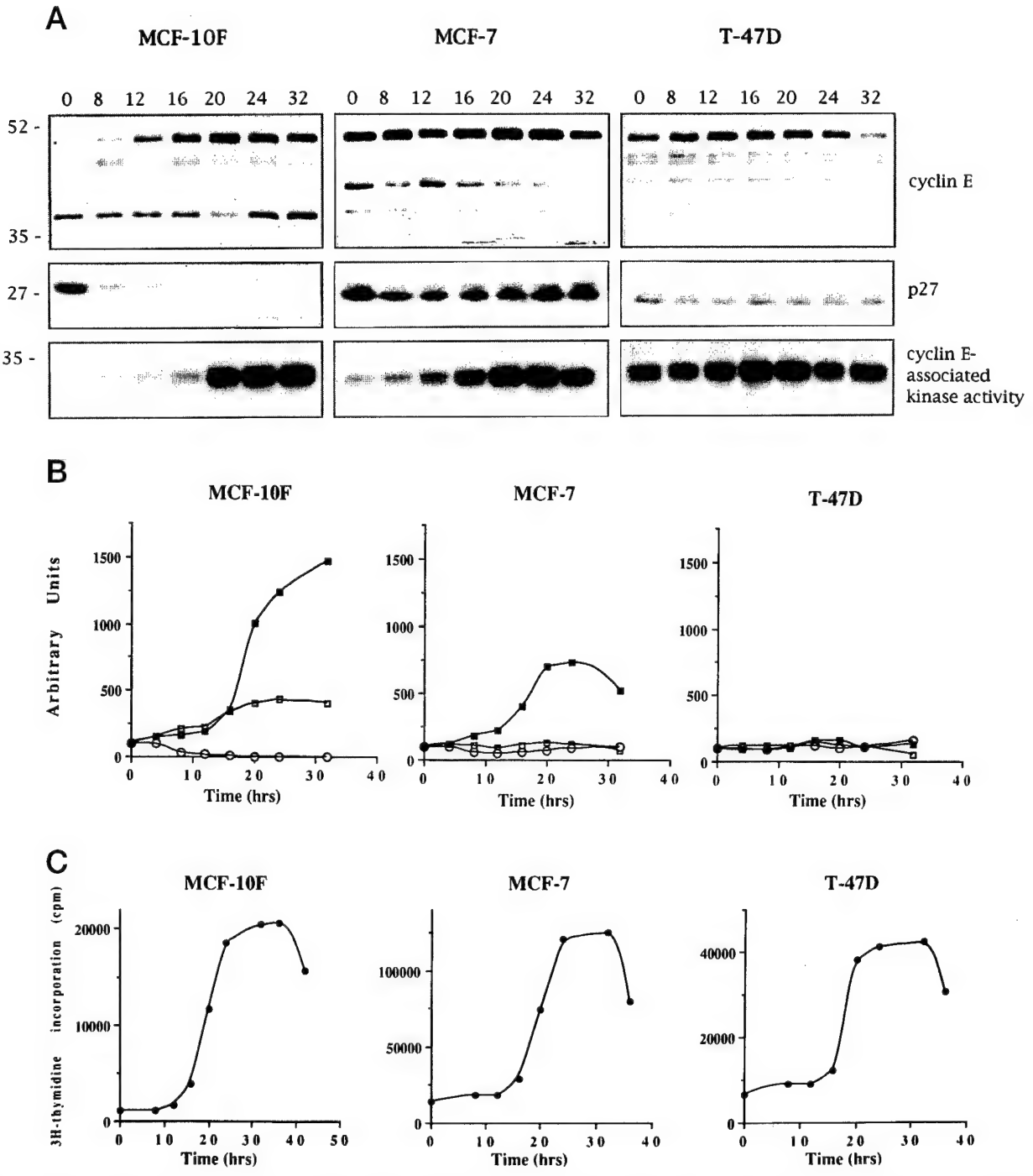

Fig. 3 Expression levels of cyclin E and $p27^{Kip1}$ and levels of cyclin E-associated kinase activity during the G_0-to-S transition in the human normal mammary epithelial cell line MCF-10F and in the breast cancer cell lines MCF-7 and T-47D. The cells were serum starved for 72 h and then stimulated to re-enter the cycle by refeeding with complete medium. *A*, whole-cell extracts were prepared from quiescent serum-starved cultures (*0*) and from cells harvested at the indicated times (in hours) after restimulation with serum. Duplicate blots were reacted with a monoclonal antihuman cyclin E antibody and a polyclonal anti-$p27^{Kip1}$ antibody, respectively, as described in the legend to Fig. 1. The film exposure times for $p27^{Kip1}$ were 3 min and 1 min, respectively, for the normal and cancer cell lines. A polyclonal anti-cyclin E antibody was used to immunoprecipitate cyclin E immunocomplexes, and kinase activity was measured by using histone H1 as a substrate. The data shown are from a representative experiment. *B*, quantitative analysis of the average of two experiments of the type shown in *A*. The intensities of each of the bands on the Western blots, as well as on the SDS-PAGE gels used to assess histone H1 kinase activity, were quantitated on a Molecular Dynamics computing densitometer using Image Quant software (version 3.22). The values are expressed as percent of the value at time 0. The same scale is used for all three cell lines to facilitate the comparison of the results. □, cyclin E; ○, $p27^{Kip1}$; ■, cyclin E-associated kinase activity. *C*, DNA synthesis in triplicate cultures of the indicated cell lines was monitored by measuring [^{3}H]thymidine incorporation into the acid-insoluble fraction after incubation for 1 h with [^{3}H]thymidine (1 μCi/ml) at the indicated times after serum addition. The data shown are from a representative experiment. For additional details, see "Materials and Methods."

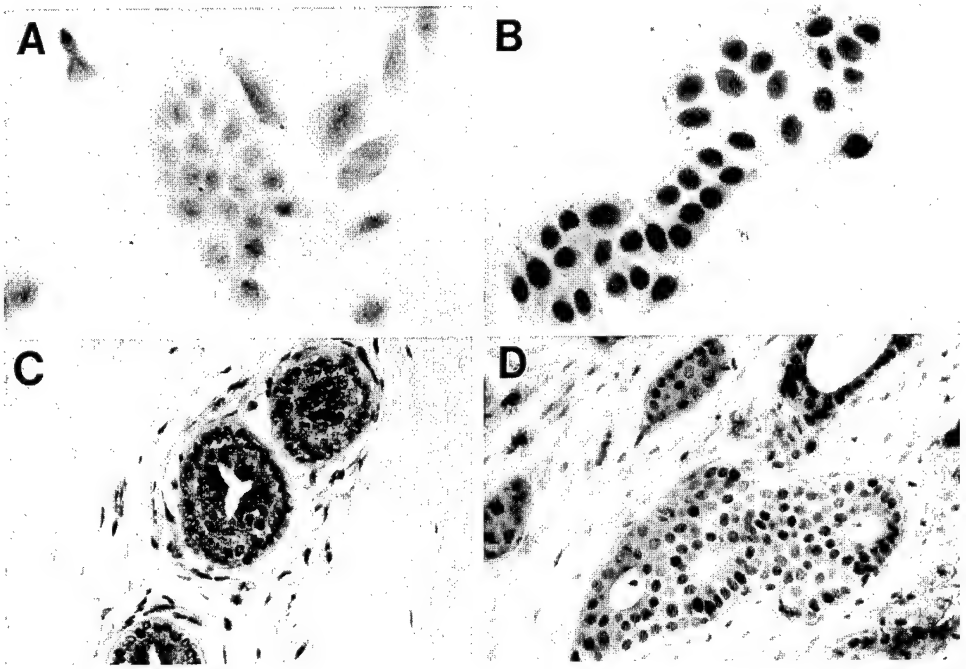

Fig. 4 Nuclear immunostaining of $p27^{Kip1}$ in cell cultures and in a primary breast cancer. Asynchronous exponentially growing MCF-10F (*A*) and MCF-7 (*B*) cells were analyzed for $p27^{Kip1}$ expression using a polyclonal anti-$p27^{Kip1}$ antibody. The same antibody was used to detect $p27^{Kip1}$ in normal breast epithelium (*C*) and in a ductal breast carcinoma (*D*). For additional details, see "Materials and Methods."

lines. The immunostaining was exclusively nuclear in both cell lines, and the relative abundance was consistent with the Western blot studies (Fig. 1), because the intensity was markedly higher in the MCF-7 cells (Fig. 4). Nuclear localization of $p27^{Kip1}$ was also seen with the 184B5, 184A1, and T-47D cell lines (data not shown).

Variable Expression of $p27^{Kip1}$ in Primary Human Breast Cancers. The above studies demonstrating deregulated expression of $p27^{Kip1}$ were obtained with breast cancer cell lines. To evaluate the significance of this finding with respect to human breast cancer, the expression of this protein was evaluated by immunostaining in a series of 52 primary human breast carcinomas (see "Materials and Methods"). When cells were positive, the immunostaining was always predominantly nuclear, as seen with the breast cancer cell lines (Fig. 4). Twenty-three of the 52 primary breast cancer samples (44%) were positive for $p27^{Kip1}$ immunostaining, *i.e.,* they displayed 2+ or 3+ intensity of staining (Table 1). Thirteen of these 23 positive cases displayed 3+ positive immunostaining. Within the group of 23 positive cases, the percentage of tumor cells within a microscopic field that were positive ranged from 10 to 80%. Even within a given tumor sample, there was often heterogeneity with respect to positive immunostaining. In the remaining 29 tumors (56%), there was very low or undetectable immunostaining for $p27^{Kip1}$ (Table 1). In 20 of the 52 tissue sections, there were sufficient normal mammary epithelial cells that could also be evaluated, and in these cases normal glands also gave 1–3+ positive immunostaining. It is of interest that, in some of the samples, the intensity of staining was higher in the tumor cells than in adjacent normal ductal epithelial cells. Normal myoepithelial cells, stromal cells, and lymphocytes were also occasionally positive (data not shown).

Data on various clinicopathological parameters including age, histology, tumor stage, nuclear grade, DNA index, S-phase fraction, estrogen and progesterone receptor levels, c-*neu*/*erb*B2 expression, lymph node involvement, and vascular invasion were available for 46 of the tumors (data not shown). $p27^{Kip1}$ immunostaining did not display a significant correlation with any of these parameters, but we emphasize that this set of samples is relatively small (Table 1).

DISCUSSION

The present study demonstrates that the level of expression of $p27^{Kip1}$ is increased in a subset of human cancer cell lines and that this increase is significantly associated with the levels of expression of cyclin D1 ($P < 0.001$) and cyclin E ($P < 0.0001$). Separate Northern blot analyses indicated that there was no correlation between the high levels of $p27^{Kip1}$ protein and the levels of the corresponding mRNA (data not shown). These results are consistent with previous evidence indicating that the expression of $p27^{Kip1}$ is mainly regulated at the protein level (32). Our results are also in agreement with our previous demonstration that ectopic overexpression of either cyclin D1 (40) or cyclin E (38) in mammary epithelial cells can lead to increased expression of the $p27^{Kip1}$ protein in the absence of changes in the corresponding mRNA,[4] although the precise mechanism is not known.

Using synchronized cell cultures, we found that the expression of $p27^{Kip1}$ is also deregulated during the cell cycle in breast cancer cell lines. Thus, whereas in the normal mammary epithelial cell line MCF-10F the level of $p27^{Kip1}$ was high in cells arrested in G_0 by serum starvation and then fell to very low levels when the cells were restimulated with serum to enter the cell cycle, in the MCF-7 and T-47D breast cancer cell lines the level of this protein remained relatively high in G_0 cells and throughout the cell cycle (Fig. 3). Parallel studies on cyclin E expression indicated that, as expected, in the MCF-10F cells the

level of this protein increased in late G_1. However, the MCF-7 and T-47D cell lines expressed a high level of multiple cyclin E bands throughout the cell cycle. The latter result is similar to the results obtained by Keyomarsi *et al.* (37) with the MDA-MB-157 breast cancer cell line, but these investigators did not examine the expression $p27^{Kip1}$. As expected, cyclin E-associated kinase activity was also cell cycle regulated in the MCF-10F cells with a peak near the onset of the S phase, coincident with the peak of expression of the cyclin E protein. Cyclin E-associated kinase activity showed no cell cycle regulation in the T-47D cells, which is consistent with the constitutive level of cyclin E in these cells. However, this kinase activity did show an increase in the MCF-7 cells during the G_0-to-S transition (Fig. 3), although this increase was not as great as in the MCF-10F cell line. The latter finding is of interest because it suggests that cyclin E-associated kinase activity might still be cell cycle regulated in some breast cancer cell lines, despite the fact that the level of expression of cyclin E remains high throughout the cell cycle. The mechanisms responsible for this regulation remain to be elucidated.

The increased expression of $p27^{Kip1}$ in several cancer cell lines was unexpected, because these cells grow rapidly and express high levels of cyclin E-associated kinase activity (37).[4] This paradox might be explained by one of three possibilities: (*a*) these cells might express a mutated form(s) of $p27^{Kip1}$ that does not inhibit Cdk activity. This seems unlikely, in view of the fact that previous attempts to find mutations of $p27^{Kip1}$ in human breast cancers were negative (24). Alternatively, post-translational modifications of $p27^{Kip1}$ in these cancer cells might inactivate its function. This also seems unlikely, because heat-inactivated protein extracts from the breast cancer cell lines MCF-7 and T-47D showed significantly higher *in vitro* CDK2 kinase-inhibitory activity than similar extracts prepared from the nontumorigenic human mammary epithelial cell lines 184B5 and MCF-10F, thus paralleling the levels of the $p27^{Kip1}$ protein in the respective cell lines (data not shown); (*b*) cyclin/Cdk complexes in tumor cells might be refractory to the inhibitory activity of $p27^{Kip1}$ due to mutations in genes that encode one (or more) of the components of the complex. This also seems unlikely, because in recent unpublished studies,[4] we have found that ectopic overexpression of $p27^{Kip1}$ in the MCF-7 breast cancer cell line does inhibit cyclin E-associated kinase activity. Moreover, a recent study has shown an inverse correlation between the levels of expression of $p27^{Kip1}$ and cyclin E- and cyclin A-associated kinase activity in primary breast cancer samples (50); and (*c*) the increased expression of $p27^{Kip1}$ might reflect the existence of a homeostatic feedback mechanism by which high levels of expression of cyclin D1 or cyclin E in some cell types leads to increased expression of $p27^{Kip1}$. This hypothesis is consistent with the correlations we found between the levels of these proteins among a series of cell lines (Figs. 1 and 2) and with our previous findings indicating that ectopic overexpression of cyclin D1 (40) or cyclin E (38) in mammary epithelial cells is associated with increased expression of $p27^{Kip1}$. Presumably, the fact that some epithelial cell lines have increased cyclin E-associated kinase activity (see above) despite the increased levels of $p27^{Kip1}$ is due to the fact that the high levels of cyclin E in these cell lines more than compensates for the inhibitory effects of $p27^{Kip1}$. It is of interest that increased expression of $p21^{Cip1}$ has been reported in primary human breast carcinomas, thus suggesting that tumor cells might also display deregulated expression of this CDI (51).

The present study also provides evidence that relatively high levels of expression of $p27^{Kip1}$ occur in a subset of primary human cancers. Therefore, our initial findings are not confined to cancer cell lines. Thus, 23 of 52 (44%) primary human breast cancers showed moderate to strong nuclear immunostaining with an anti-$p27^{Kip1}$ antibody (Table 1). It is of interest that normal breast epithelium also stained positive for $p27^{Kip1}$ and that, although 56% of the tumors expressed very low or undetectable levels of $p27^{Kip1}$, in the positive cases, the intensity of the staining in tumor cells was often higher than in the adjacent normal ductal epithelial cells.

The specificity of the immunostaining was demonstrated by always including a negative control sample in which the primary antibody was omitted. In addition, complete inhibition of the immunohistochemical staining in positive controls was obtained by preincubating the antibody with a 100-fold excess of the immunizing peptide for 1 h at room temperature (data not shown). Moreover, similar results were obtained when a different $p27^{Kip1}$ antibody (N-20, Santa Cruz) was used to stain selected cases for which duplicate slides were available (data not shown). To extend and confirm these observations, we have recently evaluated by Western blot analyses the expression levels of $p27^{Kip1}$ in a subset of primary human breast carcinomas and adjacent normal breast tissues. The $p27^{Kip1}$ protein was present at a variable level in normal mammary tissue but was increased 3–5-fold in four of the nine primary breast carcinomas examined when compared to normal adjacent tissue, and its expression level correlated with the level of expression of the cyclin E protein. Similar results were obtained with primary human colon carcinomas.[6] Studies are in progress to verify this correlation with a much larger number of cases. For the reasons mentioned above, it seems likely that the $p27^{Kip1}$ protein expressed in the primary tumors is functional, but this requires additional study.

It was of interest to search for possible correlations between $p27^{Kip1}$ expression and various clinicopathological parameters in the set of cases of breast cancer examined in the present study. No significant correlations were found between $p27^{Kip1}$ expression and various clinicopathological parameters (Table 1). We should emphasize, however, that a much larger series of cases that also provide follow-up information with respect to response to therapy and survival is required to evaluate the clinical significance of high or low expression of $p27^{Kip1}$ in our series of cases.

During the revision of this manuscript, three papers appeared in which the levels of expression of $p27^{Kip1}$ were determined by immunohistochemistry in two large cohorts of breast cancers and one of colorectal cancers (50, 52, 53). Catzavelos *et al.* (50) studied 168 primary breast cancer cases, whose mean age was 62 years. They found that $p27^{Kip1}$ expression was decreased with increasing tumor grade and that reduced $p27^{Kip1}$

[6] A. Sgambato, H. Yamamoto, M. Ciaparrone, and I. B. Weinstein, unpublished data.

expression was a strong predictor of reduced disease-free survival. Porter *et al.* (52) studied 246 young women with primary breast cancers and analyzed by immunostaining the levels of expression of both $p27^{Kip1}$ and cyclin E. They found that low or absent levels of $p27^{Kip1}$ were a strong predictor of poor outcome, especially when associated with increased levels of expression of cyclin E. In the third paper, Loda *et al.* (53) examined $p27^{Kip1}$ levels in 149 cases of primary human colorectal carcinomas. They also reported that low $p27^{Kip1}$ expression was associated with poor overall survival, particularly in stage II tumors, and that the low expression appeared to be due to an increase in $p27^{Kip1}$-ubiquitin-mediated degradation.

We find it of interest that, as in the present study (Table 1), a major fraction of the malignant tumors examined in the above three recently published studies (50, 52, 53) expressed relatively high levels of this cell cycle-inhibitory protein. Indeed, we found that some of the breast cancer cells expressed even higher levels of $p27^{Kip1}$ than adjacent normal mammary epithelial cells, although this apparently was not the case in the studies by Catzavelos *et al.* (50) and Porter *et al.* (52). However, results similar to ours were reported recently by Shuter *et al.* (54) in a subset of primary human breast cancers. In addition, a recent study on diethylstilbestrol-induced renal tumors in Syrian hamster found that the tumors expressed much higher levels of $p27^{Kip1}$ than normal renal cells (55). It is also of interest that in the present study (Table 1) and in the above-mentioned three recently published studies (50, 52, 53), there was no correlation between levels of $p27^{Kip1}$ expression and cell proliferation. Therefore, $p27^{Kip1}$ may have functions in tumor cells that are not related simply to cell cycle progression. Along these lines, it is of interest that *in vitro* the level of expression of $p27^{Kip1}$ increases when cancer cells are transferred from monolayer to three-dimensional cultures (56), and that overexpression of $p27^{Kip1}$ may be associated with increased resistance to anticancer agents (56) and to the occurrence of apoptotic cell death under three-dimensional growth conditions (57). Therefore, additional studies are required to clarify the role of $p27^{Kip1}$ in tumor biology and its clinical significance.

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Disorders in Cell Circuitry Associated with Multistage Carcinogenesis: Exploitable Targets for Cancer Prevention and Therapy[1]

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Abstract

The development of a malignant tumor involves the progressive acquisition of mutations and epigenetic abnormalities in multiple genes that have highly diverse functions. Some of these genes code for pathways of signal transduction that mediate the action of growth factors. The enzyme protein kinase C plays an important role in these events and in the process of tumor promotion. Therefore, we examined the effects of three inhibitors of protein kinase C, CGP 41251, RO 31-8220, and calphostin C, on human glioblastoma cells. These compounds inhibited growth and induced apoptosis; these activities were associated with a decrease in the level of CDC2 and cyclin B1/CDC2-associated kinase activity. This may explain why the treated cells accumulated in G_2-M. In a separate series of studies, we examined abnormalities in cell cycle control genes in human cancer. We have found that cyclin D1 is frequently overexpressed in a variety of human cancers. Mechanistic studies indicate that cyclin D1 can play a critical role in carcinogenesis because: overexpression enhances cell transformation and tumorigenesis; introduction of an antisense cyclin D1 cDNA into either human esophageal or colon cancer cells reverts their malignant phenotype; and overexpression of cyclin D1 can enhance the amplification of other genes. The latter finding suggests that cyclin D1 can enhance genomic instability and, thereby, the process of tumor progression. Therefore, inhibitors of the function of cyclin D1 may be useful in both cancer chemoprevention and therapy. We obtained evidence for the existence of homeostatic feedback loops between cyclins D1 or E and the cell cycle inhibitory protein $p27^{Kip1}$. On the basis of these and other findings, we hypothesize that, because of their disordered circuitry, cancer cells suffer from "gene addiction" and "gene hypersensitivity," disorders that might be exploited in both cancer prevention and therapy.

Introduction

It is a thrilling experience to honor J Freireich by recalling the important advances that have been made over the past few decades in the care of cancer patients. I (I.B.W.) first met J in 1957 when I was a Clinical Associate on the Metabolism Service of the National Cancer Institute and he was just beginning his pioneering studies on platelet transfusions and the chemotherapy of leukemia. I tremendously appreciate the subsequent advances in these and other areas that he spearheaded, his leadership role in clinical research, and his stimulating friendship.

This is the era of molecular genetics. To discover the molecular basis of Freireich's many talents, I wanted to analyze his genome, but I didn't have a sample of his DNA. So, I took the sequence of the letters in his name and entered it into the GenBank database on nucleic acid sequences to search for homologous sequences that might be informative. The readout I obtained is displayed in Fig. 1. It is amazing and prophetic! There is over 62% homology between the sequences Emil Freireich, M.D., and Emil Frei, M.D. In an evolutionary sense, Emil Freireich, M.D., is an extended (one might say jumbo) version of Emil Frei, M.D. We are now trying to transfer the consensus sequence into our oncology fellows so that they will acquire some of the marvelous skills shared by these two leaders in cancer therapy.

My career has emphasized research on cancer causation, with a view toward prevention. Until recently, this field seemed separate from that of cancer therapy, but in recent years, this has changed dramatically. Indeed, the fields of cancer causation, prevention, and treatment are rapidly merging, with the ultimate goals of reducing both cancer incidence and mortality. I will discuss how recent studies on disorders in signal transduction and cell cycle control, which develop during the multistage process of carcinogenesis, provide new targets for both cancer prevention and treatment. I will also discuss our hypothesis that, because of their disordered circuitry, cancer cells suffer from "gene addiction" and "gene hypersensitivity," disorders that might be exploited in cancer prevention and therapy.

It is now apparent that the development of a fully malignant tumor involves the progressive acquisition of mutations and epigenetic abnormalities in multiple genes (1, 2). Because of the large number and diverse functions of these genes, we believe that the two categories "oncogenes" and "tumor suppressor genes" are not adequate because they do not indicate the specific biochemical functions of the individual genes or con-

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Fig. 1.

sider the cellular contexts in which they function. Table 1 presents a classification scheme that attempts to achieve this goal. The genes are divided into two broad functional categories: (*a*) those that control intracellular regulatory circuitry and (*b*) those that influence cell surface and extracellular functions. The first category is further divided into four subcategories: (1) genes that play a role in the responses of cells to external growth factors, *i.e.*, genes that encode growth factors, cellular receptors, coupling proteins, and protein kinases that transduce information across the cytoplasm to the nucleus and nuclear transcription factors that then increase or repress the expression of specific genes; (2) genes involved in DNA replication and repair; (3) genes involved in cell cycle control; and (4) genes that determine cell fate, *i.e.*, cellular differentiation, senescence, and programmed cell death (apoptosis). Many of the oncogenes, for example, *ras*, are in subcategory 1. Subcategory 3 includes the tumor suppressor genes *Rb* and *p53*. Recent studies on cyclins and cyclin-related genes and their abnormalities in cancer have rapidly expanded this subcategory. This subject is discussed in greater detail below. The second category (b) includes genes that influence how cells interact with the extracellular matrix and/or neighboring cells. This category includes various cell surface proteins, cell adhesion molecules, extracellular proteases, and angiogenesis factors. Obviously, alterations in these genes are especially relevant to tumor cell invasion and metastasis. I should emphasize several caveats related to this classification scheme: some of the above-mentioned genes perform multiple functions that extend across these categories (*i.e.*, *p53*); there is cross-talk between components in each category and between categories; and the biological effects of some of these genes are dependent upon the context of the specific cell type in which it is expressed. Therefore, the classification scheme shown in Table 1 is an oversimplification. Nevertheless, it is more informative than simply using the terms oncogenes and tumor suppressor genes, and it may be useful in conceptualizing novel approaches to cancer prevention and therapy. With this theme in mind, I now want to discuss recent studies from our laboratory related to a family of genes in category a.1 of Table 1, namely, PKC,[3] and then I will turn to recent studies on genes in category a.3, namely, cyclin D1 and related genes.

Table 1 Categories of genes targeted during multistage carcinogenesis

a. Intracellular circuitry
1. Agonist-induced signal transduction
2. DNA replication and repair
3. Cell cycle control
4. Cell fate: survival, differentiation, senescence, and apoptosis
b. Cell surface and extracellular functions
Adhesion molecules; proteases; angiogenesic factors, and so on

Inhibitors of PKC

For several years our laboratory has been interested in PKC because of its central role in the action of the phorbol ester tumor promoters and related compounds and its role in mediating the action of several growth factors and oncogenes (3). Indeed, in recent studies, we demonstrated that a specific isoform, PKCε, plays a role in oncogenesis by activating the Ras/Raf/mitogen-activated protein kinase pathway of signal transduction (4). For these reasons, inhibitors of PKC might be useful in cancer prevention and treatment. Therefore, we examined, in detail, the effects of a potent inhibitor of PKC, the staurosporine derivative CGP 41251, on a series of nine human glioblastoma cell lines (5). This compound caused irreversible inhibition of the proliferation of these cell lines, with an IC_{50} of about 0.4 μM. This was associated with an increase of cells in the G_2-M phase of the cell cycle and the induction of apoptosis. These effects occurred even in cell lines carrying mutations in the *p53* gene. The treated cells displayed a decrease in the level of the CDC2 protein (also termed CDK1) and a decrease in cyclin B/CDC2-associated kinase activity. The latter effects may explain why the cells accumulated in G_2-M, but the precise mechanism remains to be determined. We obtained similar effects with another staurosporine derivative, R0 31-8220, and with the PKC inhibitor calphostin C, which acts on the regulatory rather than the catalytic domain of PKC.[4] Taken together, these findings suggest that, although these compounds are not generally thought of as cytotoxic agents, they may be effective in cancer chemotherapy because they can induce irreversible growth inhibition and apoptosis in a p53-independent manner. Indeed, CGP 41251 inhibited the growth of a human glioma cell line in nude mice (5). Therefore, we are optimistic that PKC inhibitors and other compounds that target functions in category a.1 in Table 1, for example, inhibitors of tyrosine kinase receptors and inhibitors of farnesylation of the Ras protein, might preferentially inhibit the growth of tumor cells.

Disturbances in Cell Cycle Control in Human Cancer

We will now discuss recent studies on disturbances in cell cycle control genes (category a.3 in Table 1) in human cancer and their relevance to cancer prevention and therapy. As shown in Fig. 2*A*, the orderly progression of dividing mammalian cells through G_1, S, G_2, and M is governed by a series of proteins called cyclins, which exert their effects by binding to and activating a series of specific CDKs. This process is further modulated by the phosphorylation and dephosphorylation of CDK proteins by specific protein kinases and phosphatases and

[3] The abbreviations used are: PKC, protein kinase C; CDK, cyclin-dependent serine and threonine protein kinase.

[4] M. Begemann, S. A. Kashimawo, R. M. Lunn, T. Delohery, Y-j. A. Choi, S. Kim, D. F. Heitjan, R. M. Santella, P. B. Schiff, J. N. Bruce, and I. B. Weinstein, unpublished results.

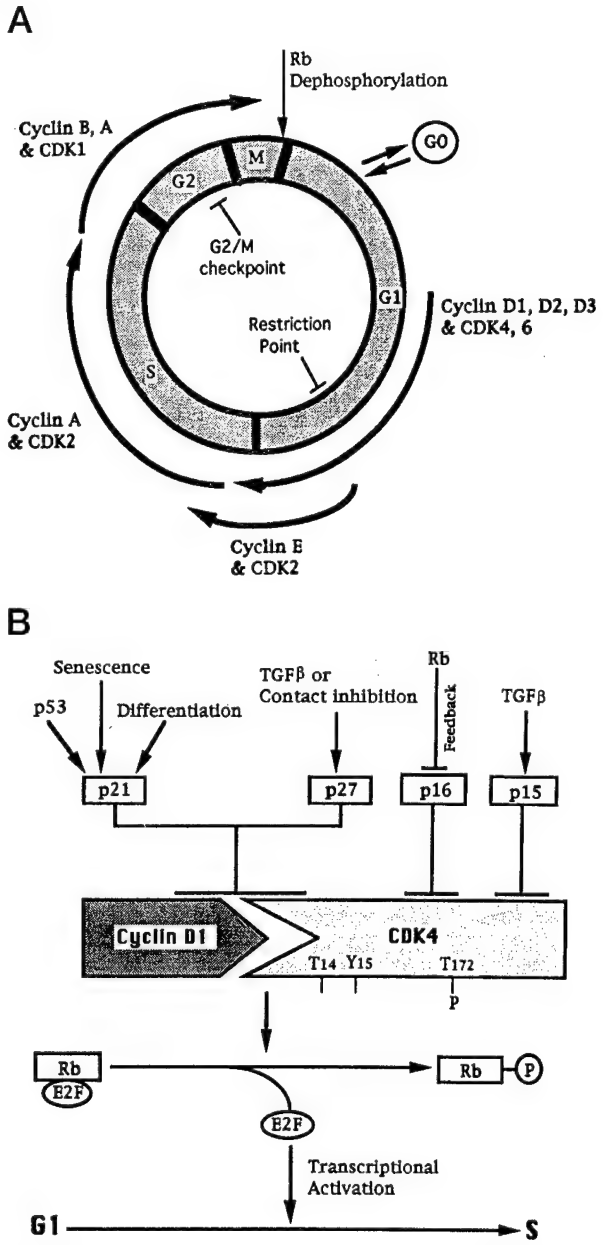

Fig. 2 *A*, simplified model of the cell cycle indicating where the major cyclin/CDK complexes act at each phase of the cell cycle. There are two checkpoints during the cell cycle: the G_1-S checkpoint (also termed restriction point), at which Rb and p53 exert inhibition on the G_1-S transition, and the G_2-M checkpoint (for additional details, see Refs. 1–3). *B*, diagram indicating the multiple mechanisms that regulate cyclin D1/CDK4 kinase (for details, see Refs. 1–3).

by a series of specific CDK inhibitor proteins called CDIs (Fig. 2*B*; Refs. 6–8).

There is accumulating evidence for mutations or abnormalities in the expression of various cyclins, CDKs, and CDIs in several types of human cancers (for review, see Refs. 6–16). The most frequent abnormalities that have been found are in the G_1 cyclin, cyclin D1. Cyclin D1 plays a key role in the G_1-S progression of the cell cycle. It binds to and activates CDK4 and CDK6. This leads to phosphorylation and inactivation of Rb, thus resulting in activation of the transcription factor E2F, which enhances S-phase progression. *cyclin D1*, also termed *prad 1* or *bcl-1*, is located at chromosome 11q13. Chromosomal rearrangements at this locus in parathyroid tumors or centrocytic B-cell lymphomas cause increased and constitutive expression of this gene. The *cyclin D1* gene is amplified and overexpressed, at both the mRNA and protein levels, in a significant fraction of primary human breast carcinomas, esophageal carcinomas, squamous cell carcinomas of the head and neck, non-small cell lung carcinomas, hepatoceullular carcinomas, and bladder carcinomas. Cytogenetic and molecular studies indicate that the amplified *cyclin D1* gene is part of a much larger amplicon located at chromosome 11q13. Overexpression of cyclin D1 in the absence of gene amplification is also seen in about 45% of human breast carcinomas (10) and about 40% of colon carcinomas (11, 12), but the mechanisms responsible for this overexpression are not known.

The increased expression of cyclin D1 could be useful in identifying preneoplastic lesions because we have found that increased expression of cyclin D1 can be detected in adenomas of the colon, *i.e.*, at a relatively early stage in the process of colon carcinogenesis (12), and also in Barrett's esophagus, a disease associated with an increased risk of esophageal cancer (13). Other investigators recently reported that cyclin D1 is also overexpressed in the small polyps of patients with familial polyposis coli and in a mouse model of this disease (14). Increased expression of cyclin D1 is a marker of poor prognosis in squamous cell carcinomas of the esophagus, squamous cell carcinomas of the head and neck (8, 15, 16), and carcinomas of the pancreas (17).

In studies on human esophageal carcinomas, we noted that the subset of tumors that had amplification and increased expression of cyclin D1 always displayed expression of the Rb protein, whereas the subset of tumors that did not express the Rb protein (presumably due to inactivating mutations or deletions) did not show amplification and increased expression of cyclin D1 (9). A similar relationship between cyclin D1 and Rb was subsequently seen in human breast (18) and non-small cell lung cancers (19). Thus, it would appear that, during the clonal evolution of tumors, the inhibitory effect of the *Rb* gene on cell cycle progression can be abrogated, either by increased expression of cyclin D1, which would increase phosphorylation of the Rb protein, thereby inactivating its inhibitory function, or actual loss of the Rb protein (9). Alternative mechanisms include inactivation of the CDI $p16^{INK4}$, which acts on cyclin D1/CDK4 and cyclin D1/CDK6. These alternative mechanisms provide a paradigm for explaining why individual tumors of the same histological type can differ with respect to the spectrum of genes that are mutated because the same regulatory pathway can be perturbed by mutations in different genes in the pathway. Therefore, in the design and clinical use of new gene-specific anticancer agents, it may be necessary to score individual tumors for the specific mutation involved or design agents that are pathway specific rather than gene specific.

Several types of mechanistic studies specifically implicate the *cyclin D1* gene in tumorigenesis. Thus, using gene transfer studies, we found that stable overexpression of cyclin D1 in

rodent fibroblasts enhanced their growth in cell culture and tumorigenicity in nude mice (20). Cotransfection studies indicated that cyclin D1 cooperates with a defective adenovirus *E1A* gene (21) or an activated *ras* oncogene (22) in the transformation of rodent cell lines. Overexpression of a *cyclin D1* sequence under the control of a mouse mammary tumor virus promoter in transgenic mice resulted in mammary hyperplasia and tumors of the mammary epithelium (23), and cyclin D1 cooperated with a *myc* oncogene in producing B-cell lymphomas in transgenic mice (24, 25). On the other hand, cyclin D1-deficient mice have reduced proliferation of the mammary epithelium (26).

Antisense to Cyclin D1 Inhibits Growth and Reverses the Transformed Phenotype of Human Esophageal and Colon Cancer Cells

Although in early studies it was known that the *cyclin D1* gene is amplified and overexpressed in a significant fraction of human esophageal tumors and several other types of human cancer, the functional significance of this overexpression had not been established. This was an important issue because, as mentioned above, the amplicon on chromosome 11q13, in which the *cyclin D1* gene resides, contains other genes, and it is possible that they, rather than cyclin D1, might be critical to tumorigenesis. To address the specific role of cyclin D1, an antisense *cyclin D1* cDNA construct was expressed, either constitutively or inducibly, in the HCE7 human esophageal cancer cell line, in which the endogenous cyclin D1 is amplified and expressed at high levels (27). The expression of antisense cyclin D1 led to decreased expression of cyclin D1 at both the mRNA and protein levels, and this was associated with a marked inhibition of cell proliferation. Antisense cyclin D1-expressing cells displayed a decreased plating efficiency, increased doubling time, decreased saturation density, increased cell size, decreased cyclin D1-associated *in vitro* Rb kinase activity, decreased anchorage-independent growth, and a loss of tumorigenicity in nude mice (27). We recently obtained similar results when an antisense *cyclin D1* cDNA was stably expressed in the SW480E8 human colon carcinoma cell line that expresses high levels of cyclin D1 in the absence of gene amplification (28). These derivatives also reverted toward normal phenotypes and lost their tumorigenicity in nude mice.

These findings provide direct evidence that the overexpression of cyclin D1 in certain tumor cells contributes to their abnormal growth and tumorigenicity. The ability to revert the transformed phenotype of these cells with antisense cyclin D1 suggests that cyclin D1 may be a useful target in cancer therapy. This could be achieved by designing antisense oligonucleotide or gene therapy approaches that would inhibit the expression of cyclin D1 in these human tumors, or, as a more feasible approach, designing drugs that inhibit the action of cyclin D1 by inhibiting the kinase function of CDK4 and CDK6.

Overexpression of Cyclin D1 Enhances Gene Amplification

A critical aspect of the multistage process of carcinogenesis is the apparent ability of tumor cells to develop genetic variants with an abnormally high frequency. Normal mammalian cells have checkpoints at the G_1-S and G_2-M stages of the cell cycle, at which cells can delay progress through the cell cycle to permit repair of damaged DNA and, thereby, prevent various types of mutations. Therefore, defects in cell cycle control and the normal function of these checkpoints might enhance genomic instability. A frequent example of genomic instability in tumors is the occurrence of gene amplification, which is often seen in cellular oncogenes and genes that play a role in drug resistance. Previous studies by other investigators demonstrated that homozygous loss of function of the *p53* gene was sufficient to increase the susceptibility of cells to gene amplification (29, 30). However, other factors can also play a role because some tumor cells with wild-type *p53* genes can still display a high frequency of gene amplification. Because *cyclin D1* plays a pivotal role in the G_1 phase of the cell cycle and this gene is frequently overexpressed in several types of human cancer, we postulated that this overexpression might contribute to genomic instability during tumor progression. Indeed, we demonstrated that ectopic overexpression of cyclin D1 in a rat liver epithelial cell line markedly increased amplification of the *CAD* gene (31). This effect was associated with impairment of G_1-S checkpoint control, although the cyclin D1-overexpressing cells had a normal *p53* gene. Overexpression of cyclin D1 also enhanced acquisition of resistance to methotrexate.[5] The capacity of cyclin D1 to enhance gene amplification could contribute to the process of genomic instability during tumor development (31). Therefore, inhibition of the action of cyclin D1 might provide a strategy for inhibiting tumor progression and the acquisition of drug resistance.

Paradoxical Expression of $p27^{KIP1}$ and Rb in Cancer Cells

During the course of our studies on the expression of various cell cycle control proteins, we were surprised to find relatively high levels of expression of $p27^{KIP1}$ and, sometimes, high levels of the Rb protein in some cancer cell lines because both of these proteins are growth inhibitors. In a series of human esophageal cancer cell lines, there was a positive correlation between the level of cyclin D1 (which would be expected to enhance growth) and levels of the $p27^{KIP1}$ and Rb proteins (32). Several human colon and breast cancer cell lines also expressed high levels of the $p27^{KIP1}$ protein, but this protein was expressed at low levels in three normal mammary epithelial cell lines (33–36). Furthermore, ectopic overexpression of cyclin D1 in an esophageal cancer cell line that expressed a low level of cyclin D1 was associated with increased expression of both $p27^{KIP1}$ and Rb (32). Ectopic overexpression of cyclin D1 or cyclin E in mammary epithelial cell lines that express low levels of both of these cyclins was also associated with increased expression of $p27^{KIP1}$ (33–35). The reciprocal effect was also seen because, when we used an antisense cyclin D1 cDNA to reduce the expression of cyclin D1 in an esophageal or colon cancer cell

[5] P. Zhou and I. B. Weinstein, unpublished results.

line, this led to reduced levels of expression of both the $p27^{KIP1}$ and Rb proteins (27, 28). Our finding that cancer cells often express high levels of the $p27^{KIP1}$ protein is not simply an artifact of cell culture because we have found that this protein is also expressed at relatively high levels in a subset of primary human breast (36) and colon tumors (37). On the basis of these findings, we postulated the existence, in some cell types, of a feedback loop between cyclins D1 or E and $p27^{KIP1}$, the function of which might be to maintain a homeostatic balance between positive and negative regulators of G_1-S progression of the cell cycle (32–36). Recent studies suggest that breast and colon tumors that display low expression of $p27^{KIP1}$ are associated with a poor prognosis (38–40); perhaps they have lost this homeostatic mechanism.

High levels of expression of another CDI, $p21^{WAF1}$, have also been seen in some human tumors, including glial tumors (41), non-small cell lung carcinomas (42), leiomyosarcomas (43), breast carcinomas (44), and pancreatic carcinomas (16). In addition, cyclin D1 can induce increased expression of $p21^{WAF1}$ through an E2F mechanism (45). These apparent paradoxes may provide another example of a homeostatic feedback mechanism that is retained in many tumors.

Gene Addiction and Hypersensitivity as Exploitable Targets

A remarkable finding in our studies using an antisense cyclin cDNA vector was that simply inhibiting the expression of cyclin D1 in either an esophageal or colon cancer cell line caused marked growth inhibition and loss of tumorigenicity, despite the fact that both cell lines are highly aneuploid and carry mutations in additional genes. Furthermore, the reverted cells still expressed appreciable levels of the cyclin D1 protein (27, 28); therefore, the growth inhibition we obtained was not simply because we blocked the expression of an essential gene. Indeed, the residual level of expression of cyclin D1 in the reverted esophageal cancer cell line was much higher than that of a tumorigenic esophageal cancer cell line in which the endogenous *cyclin D1* gene was never overexpressed (27). These findings led us to suggest that the intracellular circuitry of cancer cells that overexpress cyclin D1 requires a higher level of this protein than do cells that developed through alternative circuitry, in which this gene is not overexpressed (2, 27, 28). In this sense, the former cells are addicted to cyclin D1, which may explain the profound growth-inhibitory effects we obtained with antisense cyclin D1 in the cyclin D1-overexpressing cell lines. The above-described homeostatic feedback loop between cyclin D1 and $p27^{KIP1}$ or similar homeostatic mechanisms could be the basis for this addiction, as illustrated schematically in Fig. 3. According to this scheme, tumor cells that express high levels of cyclin D1 might also have increased levels of the inhibitory protein $p27^{KIP1}$, but cyclin D1 would be in relative excess and, thus, would cause growth stimulation. A decrease in the level of cyclin D1 in these cells could then cause marked growth inhibition because there would now be a relative excess of $p27^{KIP1}$, especially if the normal homeostatic balance between cyclin D1 and $p27^{KIP1}$ is impaired. Similar mechanisms might confer addiction to other dominant acting oncogenes in tumor cells, for example, tumor cells carrying an activated *ras* oncogene or tumor cells that express increased levels of growth factors or growth factor receptors.

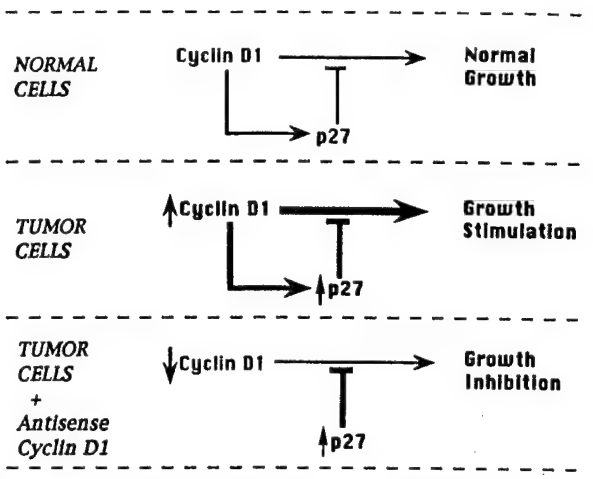

Fig. 3 Schematic diagram illustrating how tumor cells that overexpress cyclin D1 might be addicted to this protein because of increased levels of $p27^{KIP1}$.

Our ability to revert the malignant phenotype of cancer cells by altering the expression of a single gene resembles the findings by other investigators in which restoration of a single wild-type tumor suppressor gene to a cancer cell can markedly inhibit its growth and/or tumorigenicity, despite the presence of several other genetic abnormalities in the recipient cells (for examples, see Refs. 46 and 47). We postulate that the latter results also reflect abnormalities in cell circuitry in these cancer cells, such that, if they lack the expression of a tumor suppressor gene, they may be hypersensitive to the inhibitory function of that gene if it is restored. A hypothetical model based on the tumor suppressor gene *Rb* is shown in Fig. 4. In normal cells, the inhibitory functions of Rb are opposed by cyclin D1 and favored by a low level of expression of the cyclin D1/CDK4 inhibitory protein $p16^{INK4}$, thus providing a homeostatic control mechanism. Inactivation of Rb in a tumor cell is often associated with decreased expression of cyclin D1 (9, 18, 19, 48) and increased expression of $p16^{INK4}$ (49). Therefore, if a wild-type *Rb* gene is introduced into these cells, its growth-inhibitory effects could be greater than its effects in normal cells because the low level of cyclin D1 and the high level of $p16^{INK4}$ will prevent inactivation of the Rb protein by phosphorylation. The tumor suppressor gene *p53* induces the protein Mdm2, which antagonizes the action of p53 (50, 51). Disturbances in this homeostatic feedback loop in tumor cells in which the *p53* gene is inactivated might confer hypersensitivity to the restoration of wild-type p53, thus explaining the subsequent loss of tumorigenicity.

The above models related to gene addiction and gene hypersensitivity are hypothetical and probably oversimplified. Nevertheless, they may provide a rationale for new approaches to cancer prevention and therapy that exploit the bizarre circuitries of cancer cells.

Conclusion

The multistage process of carcinogenesis is associated with numerous mutations and epigenetic abnormalities in genes that

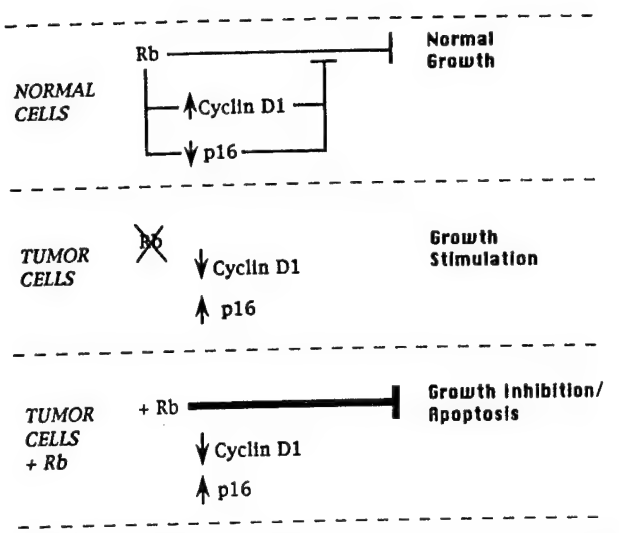

Fig. 4 Schematic diagram illustrating how tumor cells that have lost expression of the Rb protein might be hypersensitive to growth inhibition by a wild-type Rb protein because of a low level of cyclin D1 and a high level of $p16^{INK4}$.

carry out highly diverse intracellular and extracellular functions. The biological consequences and clinical significance of any single genetic abnormality in cancer cells can, therefore, only be understood within the complex network of signal transduction pathways and mechanisms of gene expression that control cellular homeostasis, cell proliferation, differentiation, and apoptosis. Recent studies on abnormalities in *cyclin D1* and related genes in human cancers illustrate these principles. This emphasis on cell circuitry and cell context has important implications in terms of targeted approaches to cancer prevention and therapy.

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Overexpression of $p27^{Kip1}$ Inhibits the Growth of Both Normal and Transformed Human Mammary Epithelial Cells[1]

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ABSTRACT

We previously reported increased expression of $p27^{Kip1}$ in a series of human breast cancer cell lines, as compared to cell lines established from normal mammary epithelial cells. These data were surprising because this protein exerts a growth-inhibitory function in normal cells, and *$p27^{Kip1}$* has been proposed as a candidate tumor suppressor gene. A possible explanation for the paradoxical increase in $p27^{Kip1}$ in the breast cancer cell lines is that they had become refractory to the inhibitory effects of this protein. To address this question, here, we transfected the MCF7 breast cancer cell line and the MCF10F nontumorigenic mammary epithelial cell line with a vector containing the $p27^{Kip1}$ cDNA to obtain derivatives that express increased levels of $p27^{Kip1}$. The increased expression of $p27^{Kip1}$ in both of these cell lines was associated with lengthening of the G_1 phase, an increase in the doubling time, a decreased saturation density, and a decreased plating efficiency. In the MCF7 cells, anchorage-independent growth and *in vivo* tumorigenicity were also suppressed. These effects were associated with decreased cyclin E-associated *in vitro* kinase activity in both cell lines. The increased expression of $p27^{Kip1}$ was associated with a decreased level of expression of cyclin D1 in the MCF10F cells but an increased level of the cyclin D1 protein in the MCF7 cell line. Both derivatives expressed slightly increased levels of the cyclin E protein. Thus, breast cancer cells are still responsive to $p27^{Kip1}$-mediated inhibition of cell growth despite the high basal level of this protein. These results suggest that therapeutic strategies that further increase the level of expression of $p27^{Kip1}$ or mimic its activity might be useful in cancer therapy.

INTRODUCTION

Cyclins control the orderly progression of cells through the cell cycle by determining the timing of activation and the substrate specificity of a series of Cdks[3] (1, 2). Both positive and negative phosphorylation events, as well as specific inhibitory proteins, play critical roles in regulating the activation of cyclin/Cdk complexes during cell cycle progression (3, 4). The Cdk inhibitors identified in mammalian cells are classified into two major categories. The INK4 family includes $p16^{Ink4a}$ (5), $p15^{Ink4b}$ (6), $p18^{Ink4c}$ (7), and $p19^{Ink4d}$ (8), which mainly inhibit Cdk4 and Cdk6 by binding to the Cdk subunit itself. The Cip/Kip family includes $p21^{Cip1}$ (9), $p27^{Kip1}$ (10), and $p57^{Kip2}$ (11), which share a conserved domain and inhibit a broader range of Cdks by binding to several cyclin/Cdk complexes. All of these Cdk inhibitors cause G_1 arrest when they are overexpressed in transfected cells (3, 12).

Loss of $p15^{Ink4b}$ and $p16^{Ink4a}$ expression due to gene mutation, deletion, and/or DNA methylation (13, 14) has been seen in a variety of human tumors, and $p16^{Ink4a}$-deficient mice develop spontaneous tumors at an early age and are highly sensitive to carcinogens (15). On the other hand, several independent studies have found that alterations in the integrity of the $p27^{Kip1}$ (16–18) and $p21^{Cip1}$ (19) genes occur only rarely in a variety of human primary tumors and cancer cell lines. Moreover, $p21^{Cip1}$-deficient mice undergo normal development and do not exhibit early tumorigenesis, although fibroblasts from these mice are defective in G_1 arrest in response to DNA damage and nucleotide pool depletion (20, 21). Mice deficient in $p27^{Kip1}$ complete an apparently normal prenatal development, although they develop hyperplasia in multiple organs, retinal dysplasia, and pituitary tumors (22–24). Nevertheless, fibroblasts from these mice display a normal G_1 arrest in response to a variety of extracellular stimuli (24).

We recently reported that ectopic overexpression of cyclin E (25, 26) or cyclin D1 (27) in a mouse mammary epithelial cell line is associated with increased expression of $p27^{Kip1}$. Increased expression of cyclin D1 (1, 2, 28) and cyclin E (29, 30) has been reported frequently in breast cancer cell lines and primary breast carcinomas. We observed that the endogenous $p27^{Kip1}$ protein is also expressed at a relatively higher level in a series of human breast cancer cell lines as compared to the level in nontumorigenic human mammary epithelial cell lines (25). The increased expression of $p27^{Kip1}$ in the breast cancer cell lines was unexpected because they grow rapidly and express high levels of cyclin E-associated kinase activity. This increase in $p27^{Kip1}$ displayed a significant association with the levels of expression of cyclin D1 and cyclin E (31) and was not confined to cancer cells *in vitro* because a high level of expression of $p27^{Kip1}$ has also been reported in a subset of primary human breast and colon carcinomas (31–33).

Possible explanations for the paradoxical increase in $p27^{Kip1}$ seen in some epithelial cancer cells are: (*a*) epithelial cells might be inherently resistant to the inhibitory effects of this protein; (*b*) epithelial cancer cells might become refractory to this protein because of mutations in one or more components of cyclin/Cdk complexes; or (*c*) epithelial cancer cells somehow sequester $p27^{Kip1}$ in an inactive form. To address this hypothesis, here, we transfected the MCF7 human breast cancer cell line with a vector containing the $p27^{Kip1}$ cDNA to obtain derivatives that stably expressed an even higher level of $p27^{Kip1}$. In parallel, we obtained derivatives of the nontumorigenic mammary epithelial cell line MCF10F that express increased levels of $p27^{Kip1}$. We found that both the MCF7 and MCF10F cells are responsive to the inhibitory effects of $p27^{Kip1}$. The implications of these findings are discussed.

MATERIALS AND METHODS

Cells and Cell Culture. The spontaneously immortalized human mammary epithelial cell line MCF10F (34) was routinely cultured in a 1:1 (v/v) mixture of DMEM and Ham's F-12 medium supplemented with 0.5 μg/ml hydrocortisone, 0.1 μg/ml cholera toxin, 10 μg/ml insulin, and 20 ng/ml epidermal growth factor (all from Sigma Chemical Co., St. Louis, MO) and 5% horse serum (Life Technologies, Inc.). The MCF7 cells were obtained from American Type Culture Collection and were cultured in MEM plus 10% FCS.

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[3] The abbreviations used are: Cdk, cyclin-dependent kinase; Rb, retinoblastoma.

Construction of Vectors and Transfection Procedure. The full-length human $p27^{Kip1}$ cDNA (1.5 kb) was subcloned into the *Eco*RI site of the eukaryotic expression vector pCDNA3 in the sense orientation. The resulting pCDNA-p27 plasmid or the control vector pCDNA3 was transfected into the MCF7 or the MCF10F cells using Lipofectin (Life Technologies, Inc.). The transfected cells were selected by growth in 700 μg/ml G418 (Boehringer Mannheim, Indianapolis, IN) for 15 days. G418-resistant colonies were then pooled, expanded, and analyzed by Western blots for $p27^{Kip1}$ expression. The pools that showed a significant increase in $p27^{Kip1}$ expressions were used for the subsequent studies.

Growth Studies. The exponential doubling times and saturation densities were determined essentially as described previously (25). Briefly, cells were plated at a density of 1×10^4 cells per 35-mm-diameter well, in triplicate. Every 2 days, the cultures were refed with fresh medium, and the number of cells per well was determined using a Coulter counter. The doubling times were calculated from the initial exponential phase of the growth curves and the saturation densities from the plateau of the growth curves. Plating efficiency assays were performed by seeding 1000 cells per 10-cm dish in complete medium. Cells were refed with fresh medium every 3–4 days for about 2 weeks. The cells were then fixed and stained with Giemsa, and the number of grossly visible colonies was counted. All assays were performed in triplicate, and all experiments were repeated at least twice and gave similar results. The data reported in Table 1 are the results of a typical experiment for each cell line.

Flow Cytometric Analysis. Exponentially growing cells were collected, washed with PBS, and fixed in 5 ml of 70% ethanol and stored at 4°C. For the analysis, cells were collected by centrifugation, and the pellets were resuspended in 0.2 mg/ml propidium iodide in Hank's balanced salt solution containing 0.6% Nonidet P-40 and RNase (1 mg/ml). The cell suspension was then filtered and analyzed for DNA content on a Coulter EPICS 753 flow cytometer. The percentage of cells in different phases of the cell cycle was determined using a ModFit 5.2 computer program. The assays were repeated at least three times and gave similar results. The data reported in Table 1 are the results of a typical experiment.

Soft Agar and Tumorigenicity Assays. To determine anchorage-independent growth, 1 ml of 0.5% agar in complete medium was placed in each 35-mm well of six well plates. Then, 2 ml of 0.3% agar in complete medium containing 1×10^5 cells were layered on top of the solidified bottom layer of agar. Colony formation was monitored for up to 3 weeks, and the final numbers of colonies that were larger than 0.05-mm diameter were determined.

Tumorigenicity assays were performed as described previously (35). Briefly, cells (5×10^6) were injected s.c. into multiple sites in athymic (nude) mice. The animals were monitored for tumor formation every week and sacrificed 2 months later. Tumor length (L) and width (W) were measured at the end of the experiment, and tumor volume was calculated by the formula $(L \times W^2)/2$.

Immunoreagents. The polyclonal antibodies to cyclin D1 and to cyclin E were obtained from Upstate Biotechnology (Lake Placid, NY). The monoclonal antibody to pRb (clone G3–245) was purchased from PharMingen (San Diego, CA). The polyclonal antibody to $p27^{Kip1}$ was from Santa Cruz Biotechnology (Santa Cruz, CA).

Protein Extraction and Immunoblotting. Proteins were extracted from exponentially growing cells and were subjected to Western blot analysis as described previously (25). Total cell lysates (50 μg) were electrophoresed by SDS-PAGE and then transferred to Immobilon-P membranes (Millipore, Bedford, MA). Appropriate dilutions were used for the different primary antibodies. Immunodetection was performed using the enhanced chemiluminescence kit for Western blotting detection (Amersham). The intensities of the bands were quantitated on a Molecular Dynamics computing densitometer (Molecular Dynamics, Sunnyvale, CA) using Image Quant software version 3.22. Protein extraction was independently performed at least three times for each cell line. Similar results were obtained in replicate studies.

***In Vitro* Assays for Cyclin E-associated Kinase Activity.** Assays for cyclin E-associated histone H1 kinase activity were performed as described previously (25). Immunoprecipitations were carried out with 2 μg of the polyclonal anti-cyclin E antibody, and immunocomplexes were recovered with protein A-Sepharose. The final pellet was resuspended in 30 μl of kinase buffer supplemented with 2 μg of histone H1 (Boehringer Mannheim) and 5 μCi of [γ-^{32}P]ATP (Amersham), and this assay mixture was then incubated for 15 min at 30°C. The reaction was stopped by addition of 25 μl 2 x-concentrated Laemmli sample buffer. The samples were separated by SDS-PAGE, and the phosphorylated histone H1 was visualized by autoradiography. The intensities of the bands were quantitated by densitometric scanning. Protein extractions and immunocomplex kinase assays were independently performed at least three times for each cell line, and repeated assays gave similar results.

RESULTS

Transfection and Expression of an Exogenous $p27^{Kip1}$ cDNA in the MCF10F and MCF7 Cells. The MCF10F nontumorigenic human mammary epithelial cell line and the MCF7 human breast cancer-derived epithelial cell line were transfected with the pCDNA3 mammalian expression vector or its derivative pCDNA-p27, containing a full-length human $p27^{Kip1}$ cDNA sequence in the sense orientation. Following G418 selection, pools of thousands of resistant colonies were obtained both from the cultures transfected with the pCDNA-p27 construct and the cultures transfected with the pCDNA3 vector (vector control cells) and analyzed for $p27^{Kip1}$ expression. Expression of the exogenous $p27^{Kip1}$ was verified by Western blot analysis using a polyclonal antibody to $p27^{Kip1}$. Representative data are shown in Fig. 1, *A* and *B*, for the MCF10F and the MCF7 cell lines, respectively.

On the basis of these results, we chose the following derivatives of MCF10 cells: one vector control pool (MCF10F-PV1) and two $p27^{Kip1}$ overexpressing derivatives (MCF10F-PKIP#1 and MCF10F-PKIP#2) for further studies. Densitometric analysis of Western blots

Table 1 *Effects of $p27^{Kip1}$ overexpression on cell cycle distribution and growth properties in normal human mammary epithelial (MCF10F) and human breast cancer (MCF7) cell lines*

Cell line	D.T.[a] (h)	S.D. ($\times 10^6$)	P.E. (%)	Cell cycle phase[b] G_0/G_1 (%)	S (%)	G_2/M (%)	A.I.G. (%)
MCF10F-PV#1	29.4 ± 2	2.9 ± 0.4	6.2 ± 2	60.5	18.8	20.7	0
MCF10F-PKIP#1	32.6 ± 2	1.2 ± 0.2	2.1 ± 1	66.6	14.4	19.0	0
MCF10F-PKIP#2	34.4 ± 3	0.3 ± 0.05	0.6 ± 0.1	71.8	10.7	19.5	0
MCF7-PV#1	28.4 ± 2	4.2 ± 0.4	33 ± 6	41.2	35.9	22.9	17 ± 3
MCF7-PV#2	27.2 ± 2	4.8 ± 0.7	27 ± 5	34.6	43.3	22.1	17 ± 3
MCF7-PV#3	29.2 ± 3	4.3 ± 0.6	26 ± 3	48.6	34.8	16.5	18 ± 2
MCF7-PKIP#9	35.9 ± 6	3.6 ± 0.5	21 ± 6	50.5	35.7	13.8	11 ± 2
MCF7-PKIP#14	37.9 ± 3	3.6 ± 0.3	10 ± 2	59.5	29.1	11.4	8 ± 1
MCF7-PKIP#23	37.2 ± 3	3.5 ± 0.4	9 ± 1	60.2	24.6	15.2	5 ± 1
MCF7-PKIP#45	39.6 ± 4	2.4 ± 0.3	7 ± 1	62.4	25.1	12.5	1 ± 1

[a] D.T., doubling time, corresponds to the initial exponential phase of cell growth; S.D., saturation density, represents the total number of cells per 35-mm well when the cultures reached a plateau in their growth; P.E., plating efficiency; A.I.G., anchorage-independent growth, expressed as colony-forming efficiency in soft agar. Values for D.T., S.D., P.E., and A.I.G. indicate the means ± the SDs ($n = 3$).

[b] Exponentially growing cultures of the indicated cell lines were analyzed by flow cytometry. The values represent the percentage of the total cell population in each phase of the cell cycle. Similar results were obtained in a repeat experiment; for additional details, see "Materials and Methods."

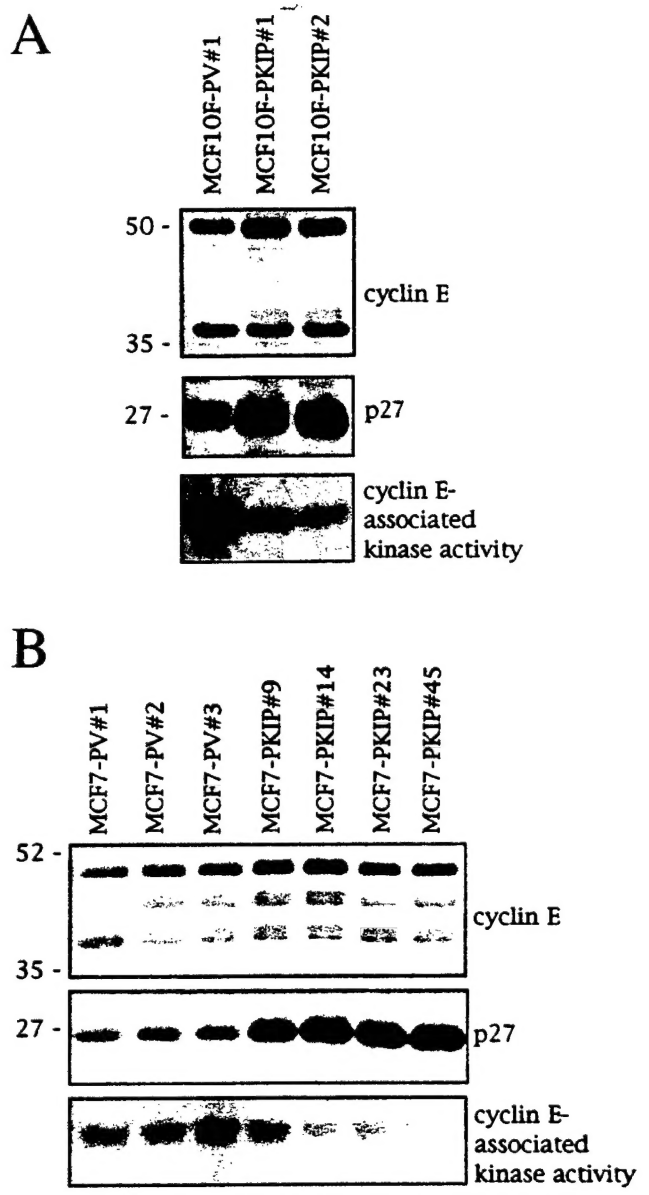

Fig. 1. Effects of ectopic expression of a human p27^{Kip1} cDNA on cellular levels of the p27^{Kip1} and cyclin E proteins and on *in vitro* cyclin E-associated kinase activity in the MCF10F (*A*) and MCF7 (*B*) cell lines. Exponentially growing cultures of vector control (*-PV*) and p27^{Kip1}-overexpressing pools (*-PKIP*) of MCF10F and MCF7 were analyzed. For Western blot analyses, 50 μg of protein in whole cell lysates were resolved by 10% SDS-PAGE and then transferred to an Immobilon membrane. Duplicate blots were probed with anti-cyclin E (*top*) and anti-p27^{Kip1} (*middle*) antibodies, and immunoreactive bands were detected by enhanced chemiluminescence. Fifty μg of whole cell lysates were also assayed for cyclin E-associated histone HI kinase activity (*bottom*). For additional details, see "Materials and Methods."

indicated that the MCF10F-PKIP#1 and MCF10F-PKIP#2 pools expressed 2- and 2.5-fold increases in p27^{Kip1} expression, respectively, as compared to the vector control pool MCF10F-PV1 (Fig. 1A). Three vector control pools (MCF7-PV#1, MCF7-PV#2, and MCF7-PV#3) and four p27^{Kip1}-overexpressing pools (MCF7-PKIP#9, MCF7-PKIP#14, MCF7-PKIP#23, and MCF7-PKIP#45) of the MCF7 derivatives were chosen for the studies described below. Densitometric analysis of Western blots indicated that p27^{Kip1} expression was increased ~4-, 6-, 6-, and 8-fold, respectively, in the MCF7-PKIP#9, MCF7-PKIP#14, MCF7-PKIP#23, and MCF7-PKIP#45 derivatives, as compared to the corresponding vector control pools (Fig. 1*B*). The increased expression of p27^{Kip1} in the derivatives of both the MCF10F and MCF7 cell lines was verified by immunostaining with an anti-p27^{Kip1} antibody. The immunostaining also confirmed the nuclear localization of the p27^{Kip1} protein in both the vector control and overexpresser derivatives (data not shown).

Effects of p27^{Kip1} Overexpression on Cell Cycle Kinetics and Cell Growth. Cell cycle parameters were examined in exponentially growing cultures of the MCF10F-PV#1 vector control and p27^{Kip1}-overexpressing cells by flow cytometry. As expected, the two p27^{Kip1}-overexpressing pools displayed an increase in the percentage of cells in the G_1 phase of the cell cycle (~69 *versus* 60%) and a reduction in the percentage of cells in the S phase (~12 *versus* 19%), as compared to the vector control pools (Table 1). In addition, the p27^{Kip1}-overexpressing cells displayed a slightly longer doubling time and a marked decrease in saturation density and plating efficiency, as compared to the vector control cells (Table 1 and Fig. 2*A*). Overexpression of p27^{Kip1}, however, did not alter the morphology of the MCF10F cells (data not shown).

Overexpression of p27^{Kip1} also did not alter the morphology of the MCF7 cells (data not shown). The p27^{Kip1}-overexpressing pools of the MCF7 cells showed an increase in the percentage of cells in the G_1 phase of the cell cycle (~58 *versus* 41%) and a decrease in the percentage of cells in the S phase (~29 *versus* 38%), as compared to the corresponding vector control cells (Table 1). The overexpressing derivatives of the MCF7 cells also displayed a longer doubling time, a reduced saturation density, and a decreased plating efficiency, as compared to the corresponding vector control cells (Table 1 and Fig. 2*B*).

Overexpression of p27^{Kip1} Reduces Anchorage-independent Growth and Tumorigenicity in the MCF7 Cells. As shown in Table 1, when grown in suspension in 0.3% agar in complete medium, the vector control MCF7-PV cells formed large colonies with a colony-forming efficiency of ~17%. In contrast, the four p27^{Kip1}-overexpressing pools displayed a decreased cloning efficiency in soft agar, with a value of ~6%, and the average colony size was smaller than that seen with the vector control cells (data not shown). These experiments were repeated three times and gave similar results.

The tumorigenicity of the vector control MCF7-PV cells and the MCF7 p27^{Kip1}-overexpressing cells were assayed in nude mice by s.c. injection of 5×10^6 cells/injection site. The MCF7-PV cells produced tumors at all of the injection sites ($n = 12$). The MCF7-PKIP cells produced tumors in all but one of of the injection sites (15 of 16). However, the latency period before tumors were detectable was longer for the p27^{Kip1}-overexpressing cells (~4 *versus* 2 weeks; data not shown), and the average tumor size was significantly smaller ($P < 0.007$), as compared to the vector control cells (Fig. 3). The parental MCF10F cells do not form colonies in agar and are not tumorigenic in nude mice; therefore, derivatives of these cells were not assessed for these parameters.

Effects of p27^{Kip1} Overexpression on the Expression and Activity of Other Cell Cycle-related Genes. Although p27^{Kip1} is found in various cyclin/Cdk complexes throughout the G_1 phase of the cell cycle, it appears that the cyclin E/Cdk2 complex is the major target of its inhibitory activity (3, 4, 10). Therefore, we examined the effects of p27^{Kip1} overexpression on *in vitro* cyclin E-associated kinase activity in both the MCF10F and MCF7 derivatives. As expected, cyclin E-associated kinase activity was markedly decreased in the MCF10F-PKIP cells, as compared to the MCF10F-PV vector control cells (Fig. 1*A*). This was also true of Cdk2-associated kinase activity (data not shown). Densitometric analysis indicated that the cyclin E-associated kinase activity was decreased to ~25 and 15% of the value in the control cells in the MCF10F-PKIP#1 and MCF10F-PKIP#2 pools, respectively, Interestingly, Western blot analysis of exponentially dividing cells indicated that the levels of expression of the cyclin E protein were slightly increased in the p27^{Kip1} overexpresser cells

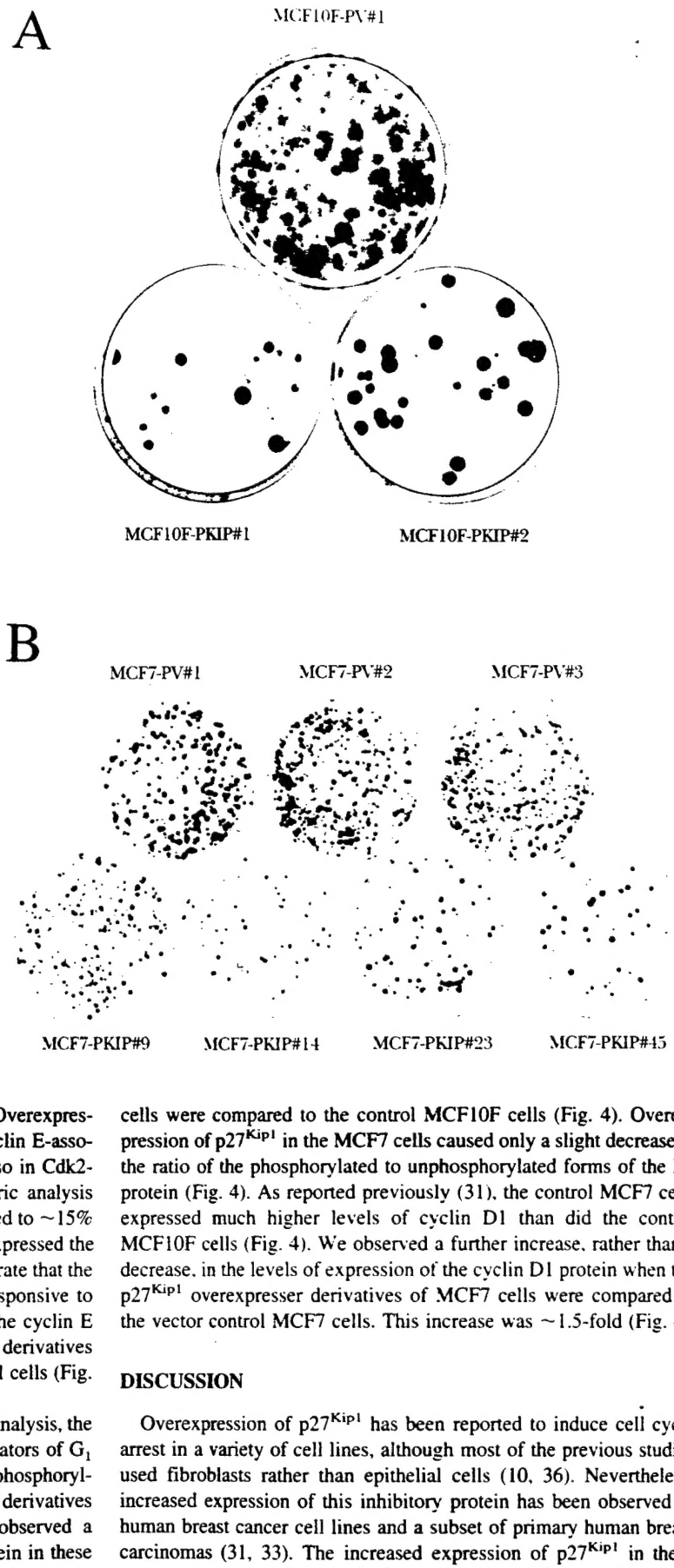

Fig. 2. Effects of stable overexpression of p27^{Kip1} on the plating efficiency of the MCF10F (*A*) and MCF7 (*B*) cells. Vector control (*-PV*) and p27^{Kip1}-overexpressing derivatives (*-PKIP*) were seeded in triplicate in 10-cm dishes (10^3 cells/dish) in complete medium and were refed with fresh medium every 3–4 days. The cells were fixed and stained with 5% Giemsa after 2–3 weeks, and the number of grossly visible colonies was counted.

(~1.4-fold), as compared to the control cells (Fig. 1*A*). Overexpression of p27^{Kip1} was also associated with a decrease in cyclin E-associated kinase activity in the MCF7 cells (Fig. 1*B*) and also in Cdk2-associated kinase activity (data not shown). Densitometric analysis indicated that the cyclin E-associated activity was decreased to ~15% of the control value in the MCF7-PKIP#45 pool, which expressed the highest level of exogenous p27^{Kip1}. These results demonstrate that the cyclin E/Cdk2 complex in this cancer cell line is still responsive to p27^{Kip1}-mediated inhibition. The level of expression of the cyclin E protein was also increased in the p27^{Kip1}-overexpressing derivatives of the MCF7 cells (~1.5-fold), as compared to the control cells (Fig. 1*B*).

To extend these results, we evaluated, by Western blot analysis, the effects of p27^{Kip1} overexpression on two additional regulators of G_1 progression. As expected, we observed a decrease in the phosphorylated form of the Rb protein in the p27^{Kip1}-overexpressing derivatives of the MCF10F cells (Fig. 4). Surprisingly, we also observed a decrease in the levels of expression of the cyclin D1 protein in these derivatives. Densitometric analysis indicated an ~50% reduction in the level of the cyclin D1 protein when the p27^{Kip1} overexpressing cells were compared to the control MCF10F cells (Fig. 4). Overexpression of p27^{Kip1} in the MCF7 cells caused only a slight decrease in the ratio of the phosphorylated to unphosphorylated forms of the Rb protein (Fig. 4). As reported previously (31), the control MCF7 cells expressed much higher levels of cyclin D1 than did the control MCF10F cells (Fig. 4). We observed a further increase, rather than a decrease, in the levels of expression of the cyclin D1 protein when the p27^{Kip1} overexpresser derivatives of MCF7 cells were compared to the vector control MCF7 cells. This increase was ~1.5-fold (Fig. 4).

DISCUSSION

Overexpression of p27^{Kip1} has been reported to induce cell cycle arrest in a variety of cell lines, although most of the previous studies used fibroblasts rather than epithelial cells (10, 36). Nevertheless, increased expression of this inhibitory protein has been observed in human breast cancer cell lines and a subset of primary human breast carcinomas (31, 33). The increased expression of p27^{Kip1} in these cancer cells is especially intriguing because several studies have also indicated that the *p27^{Kip1}* gene is usually not mutated in breast

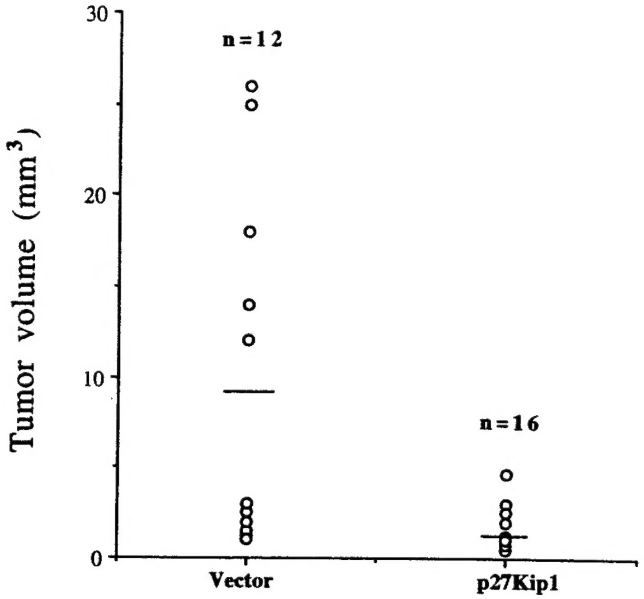

Fig. 3. Effects of stable overexpression of p27^{Kip1} on the tumorigenicity of the MCF7 cells in nude mice. Cells (5×10^6) from exponentially dividing cultures of each vector (MCF7-PV#1, MCF7-PV#2, and MCF7-PV#3) and p27^{Kip1}-overexpressing pools (MCF7-PKIP#9, MCF7-PKIP#14, MCF7-PKIP#23, and MCF7-PKIP#45) were injected s.c. into multiple ($n = 4$) sites in athymic (nude) mice. The animals were monitored for tumor formation every week and sacrificed 2 months later. Tumor length (L) and width (W) were measured at the end of the experiment, and tumor volume was calculated by the formula ($L \times W^2$)/2. The Mann-Whitney test was used to determine the statistical significance between the mean tumor volumes (—) of the vector control and p27^{Kip1} overexpressing pools ($P < 0.007$).

cancers, and therefore, they overexpress a wild-type protein (18, 37, 38).

We have previously hypothesized that the increased levels of p27^{Kip1} in breast cancer cells might reflect the existence of a homeostatic regulatory mechanism that protects the cancer cells from potentially toxic effects of increased expression of cyclin E and/or cyclin D1 (25, 26). Indeed, we have observed a significant association between increased expression of p27^{Kip1} and cyclin E or cyclin D1 in human breast cancer cell lines (31). The paradoxical increase in p27^{Kip1} in breast cancer cells might indicate that mammary epithelial cells are inherently resistant to the inhibitory effects of p27^{Kip1} or that cyclin/Cdk complexes in breast cancer cells are refractory to the inhibitory activity of p27^{Kip1} due to mutations in one (or more) components of these complexes. Therefore, it was of interest to examine the phenotypic effects of expressing an exogenous p27^{Kip1} in both normal and tumor-derived human mammary epithelial cells.

This study demonstrates that, as reported previously for other cell types, overexpression of p27^{Kip1} does inhibit the growth of normal human mammary epithelial cells. In fact, overexpression of an exogenous p27^{Kip1} cDNA in the normal nontumorigenic human mammary epithelial cell line MCF10F was associated with lengthening of the G_1 phase of the cell cycle, a longer doubling time, a decreased saturation density, and a decreased plating efficiency (Table 1 and Fig. 2*A*). As expected, these phenotypic effects were associated with a marked inhibition of cyclin E-associated kinase activity (Fig. 1*A*).

Despite its rapid *in vitro* growth and *in vivo* tumorigenicity, MCF7 cells express a high level of p27^{Kip1} (26, 31). Nevertheless, a further increase in the cellular level of p27^{Kip1} due to ectopic expression of p27^{Kip1} was also able to inhibit the growth of this cell line. Thus, the p27^{Kip1}-overexpressing derivatives of the MCF7 cells displayed an increase in the percentage of cells in the G_1 phase of the cell cycle, a longer doubling time, a decreased saturation density, and a decreased plating efficiency (Table 1 and Fig. 2*B*). Furthermore, the anchorage-independent growth and the *in vivo* tumorigenicity of MCF7 cells were significantly reduced, although not completely abolished (Table 1 and Fig. 3), by the increased expression of p27^{Kip1}. Cyclin E-associated kinase activity was also markedly decreased in these derivatives, as compared to vector control MCF7 cells (Fig. 1*B*).

Overexpression of p27^{Kip1} had different effects on the levels of expression of the cyclin D1 protein in the normal MCF10F and MCF7 breast cancer cell lines. The levels of the cyclin D1 protein were reduced in the p27^{Kip1}-overexpressing derivatives of the MCF10F cells, as compared to the vector control MCF10F cells (Fig. 4). The mechanism responsible for this decrease is not known. It could simply reflect the growth inhibition seen in these derivatives. However, the level of the cyclin E protein was slightly increased in the p27^{Kip1}-overexpressing MCF10F cells (Fig. 1*A*). The latter effect might reflect an attempt of the cells to override the growth inhibition caused by the increased level of p27^{Kip1}. It might also simply be a consequence of reduced turnover of the cyclin E protein because cyclin E is degraded following its phosphorylation by the cyclin E/Cdk2 complex (39), and this kinase activity is inhibited in these cells (Fig. 1*A*).

Curiously, overexpression of p27^{Kip1} in the breast cancer-derived MCF7 cells was associated with increased levels of both the cyclin D1 and cyclin E proteins (Figs. 1*B* and 4). The increased level of cyclin E might, again, be a consequence of reduced turnover due to reduced phosphorylation by the cyclin E/Cdk2 complex. More intriguing is the increased expression of cyclin D1 because this did not occur in the derivatives of MCF10F cells (Fig. 4). The increased expression of cyclin D1 and cyclin E in the p27^{Kip1}-overexpressing derivatives of MCF7 cells may also reflect an adaptive mechanism in which the tumor cells attempt to override the growth-inhibitory activity of the increased level of p27^{Kip1}. Indeed, increased expression of cyclin E has been shown to rescue cells from p27^{Kip1}-mediated growth inhibition (40). Western blot analyses failed to detect any change in the levels of expression of cyclin A, Cdk2, Cdk4, or p21^{Cip1} in the p27^{Kip1}-overexpressing derivatives of MCF10F or MCF7 cells (data not shown).

We previously observed a reciprocal phenomenon, in which ectopic overexpression of cyclin D1 or cyclin E in mammary epithelial cells is associated with increased expression of p27^{Kip1} (25–27). Increased levels of endogenous cyclin D1 and cyclin E also correlate with increased levels of p27^{Kip1} in breast cancer cell lines (25, 31). Furthermore, there is a significant association between the levels of p27^{Kip1} and cyclin D1 in primary breast (33) and colon (32, 33) carcinomas and also in esophageal cancer cell lines (41, 42). It is not clear whether the increase in cyclins D1 and E or the increase in p27^{Kip1} occurs first during tumor development.

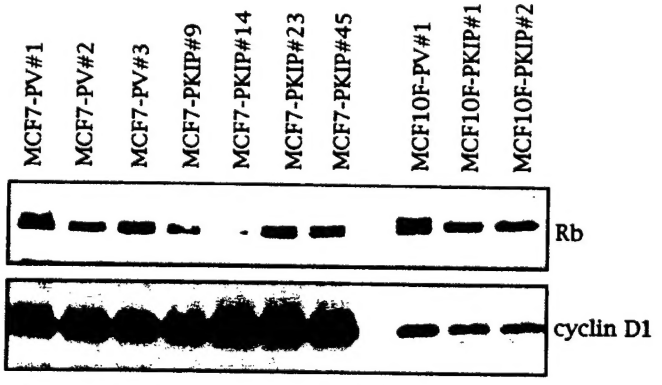

Fig. 4. Effects of stable overexpression of p27^{Kip1} on the levels of expression of cyclin D1 and Rb proteins in the derivatives of the MCF10F and MCF7 cells. Extracts were prepared from exponentially dividing cultures and examined by Western blot analysis, as described in the legend to Fig. 1 and "Materials and Methods."

Taken together, these findings provide evidence for the existence in mammalian epithelial cells of a complex homeostatic network of feedback loops that maintains an optimum balance between positive and negative regulators of the G_1 to S transition of the cell cycle. It appears that at least some components of this network can be retained in breast cancer cells, presumably because this provides a growth and/or survival advantage. However, here we demonstrate that tumor cells are unable to counteract a marked or abrupt increase in the level of expression of $p27^{Kip1}$. In fact, despite the increased expression of cyclin D1 and cyclin E, the $p27^{Kip1}$-overexpressing derivatives of MCF7 cells were inhibited in both their growth and tumorigenicity (Table 1 and Fig. 4). These results suggest that strategies that would increase cellular levels of $p27^{Kip1}$ or mimic its effects (43) might be useful in cancer therapy, because they would inhibit the growth and tumorigenicity of cancer cells, even in the presence of high endogenous levels of cyclin D1 and/or cyclin E. Our findings are consistent with recent data obtained by transient ectopic overexpression of $p27^{Kip1}$ using a replication-incompetent adenovirus delivery system in human breast cancer (44) and brain tumor (45) cells. It appears that $p27^{Kip1}$ is more effective than $p21^{Cip1}$ in inducing cell cycle arrest and Cdk2 inhibition in tumor cells (44). Several growth-inhibitory factors, such as transforming growth factor-β (36, 46), contact inhibition (47), IFN-γ (48), IFN-β (49), cAMP (50), and rapamycin (51) may function, at least in part, by inducing the expression or enhancing the activity of $p27^{Kip1}$. These observations, together with the results of the present study, suggest novel strategies for the therapy of cancer.

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